DETERMINING A ROLE FOR CD45 IN DENDRITIC CELLS

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Abstract

CD45 is a leukocyte specific protein tyrosine phosphatase present on the surface of all nucleated, hematopoietic cells. Despite its well-characterized role in antigen receptor signaling, little is known about its function in cell types like dendritic cells (DCs). DCs are crucial to the immune response both for its initiation and for its suppression. In this dissertation, the effects of the lack of CD45 on dendritic cell development and function were studied.

The most important finding was that the lack of CD45 had a differential impact on the proinflammatory cytokine profiles elicited in DCs by different TLR agonists. TLR4 ligation led to a decrease in proinflammatory cytokine and IFNβ production whereas stimulation through TLR2 or TLR9 increased cytokine production. This suggests CD45 may be acting as a negative regulator of MyD88-dependent cytokine signaling and a positive regulator of the Trif pathway.

The absence of CD45 caused alterations in the phosphotyrosine levels of several Src family kinases including Lyn. In $CD45^{-/-}$ DCs, Lyn was not activated upon LPS stimulation and several substrates of Lyn that appear as negative regulators in the MyD88-dependent pathway of TLR4 signaling are also not phosphorylated, providing evidence that CD45 may be a negative regulator of this pathway.

The absence of CD45 in TLR activated DCs had an effect on the IFNγ secretion by CD4+ T cells and NK cells, consistent with the cytokine profiles of the DCs These data demonstrate that modulation of TLR signaling by CD45, in DCs, has the ability to impact the development of the adaptive immune response.

The absence of CD45 in mice did not result in increased survival upon challenge with a high dose of LPS. Serum TNFα levels were increased in the $CD45^{-/-}$ mice and they showed more severe symptoms of septic shock. However, the $CD45^{-/-}$ mice were also found to have an increase in the number of peritoneal macrophages.

Overall this study shows that CD45 does play an important role in cell types other than lymphocytes. CD45 is a regulator of TLR-mediated cytokine secretion in DCs and thus directs the outcome of the adaptive immune response.

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LIST OF ABBREVIATIONS

Ab	Antibody
APC	Antigen presenting cell
BCR	B cell receptor
BMDC	Bone marrow-derived dendritic cell
BSA	Bovine serum albumin
cDC	Conventional DC
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
EDTA	Ethylene-diamine-tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FcR	Fc receptor
FCS	Fetal calf serum
HBSS	Hanks balanced salt solution
HRP	Horseradish peroxidase
ICAM	Intracellular adhesion molecule
IFNAR	Interferon α/β receptor
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif

LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
NK	Natural killer
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositide-3-kinase
PMA	Phorbol myristate acetate
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
PVDF	Polyvinylidine fluoride
RPMI	Rosewell Park Memorial Institute-1640 medium
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFK	Src-family kinase
SH	Src-homology
SHIP	Src-homology-domain containing 5'-inositol phosphatase
TCR	T cell receptor
Th	T-helper
Treg	Regulatory T cell

1.1 Overview of dendritic cells and the immune system

The innate immune system is the first line of defense against invading microorganisms and foreign substances. Whereas the adaptive immune response requires several days for its full induction, the innate immune response is effective within minutes of exposure. Although the adaptive response is precise and can recognize a vast number of specific antigens, the innate response relies largely on the expression of germ-line encoded, invariant receptors that evolved to recognize the distinct signature caused by states of disease. These receptors have been coined pattern-recognition receptors (PRRs) because the molecules they recognize are evolutionarily conserved and associated with microbial pathogens or cellular stress. The classic example of this is bacterial lipopolysaccharide (LPS). PRRs allow the cells of the innate system to recognize a broad spectrum of pathogens and also allow them to differentiate between healthy and diseased states.

Components of the innate immune system include the skin and mucous membranes, physiological barriers (such as the acid pH of the stomach), secreted proteins (such as complement) and phagocytic cells (such as polymorphonuclear leukocytes and macrophages). Components of the adaptive immune response include T and B lymphocytes. In higher mammals, the innate and adaptive immune systems work together in a highly coordinated and well-orchestrated fashion. An important link between the two are professional antigen presenting cells (APCs), the most important of which are the dendritic cells.

Dendritic cells (DCs) are the only APCs that can directly activate naïve T cells. DCs are the sentinels of the immune system and are largely distributed in peripheral tissues where

they exist in a non-immunogenic state called “immature”. An immature DC can be characterized by low levels of costimulatory molecule expression along with a high capacity to take up environmental antigens through mechanisms like phagocytosis, endocytosis and macropinocytosis. When a DC senses a pathogen through their PRRs they go through a maturation process that makes them highly functional T cell activators and allows them to traffic to the lymph nodes where T cells reside.

In addition to activating T cells, DCs are also charged with preventing T cells from inappropriately recognizing and attacking self. DCs play an important role in central tolerance by deletion of self-reactive $CD4^+$ $CD8^+$ thymocytes *in vivo*. In the periphery, migration of semi-mature DC expressing low-levels of T cell stimulatory molecules to lymph nodes may maintain self-tolerance either through deletion of reactive T cells or by induction of regulatory T cells. Thus, DCs are a vital link between innate and adaptive immunity and are pivotal in directing an appropriate response from the adaptive immune system.

1.2 Dendritic cells

1.2.1 Dendritic cell subsets

DCs are a heterogeneous population that can be distinguished based on differential expression of surface markers. The basic functions of antigen uptake, processing and presentation to naïve T cells are common to all DC but their anatomical location, responses to stimuli and the resulting adaptive immune response they prime are different. In the steady state, DC can be very broadly divided into two classes: conventional DCs (cDCs) and

plasmacytoid DC (pDC). Conventional DCs can be further subdivided into migratory DC, which are located in the periphery and migrate to the lymph nodes carrying peripheral antigens to present to T cells, and lymphoid-tissue-resident DCs, which acquire and present antigens localized only in the lymphoid organ where they reside. The most classical example of migratory DCs are the Langerhans cells and an example of lymphoid-resident DCs are splenic DCs.

Lymphoid-resident DCs are a heterogeneous population consisting of specialized subsets that can be identified based on the differential expression of several surface markers. In the mouse, the most classical division of these DCs uses CD8α and CD11b as markers for "lymphoid" and "myeloid" DCs respectively. The use of these terms is no longer considered to denote their hematopoietic origin as both subpopulations can be obtained *in vivo* by reconstitution of mice with either common-myeloid or common-lymphoid progenitors (fig 1.1). More recent work has associated the $CD8\alpha^+$ subset with the surface antigen, DEC-205 and the $CD8\alpha^-CD11b^+$ subset with the dendritic cell inhibitory receptor-2 (DCIR2) recognized by the monoclonal antibody, 33D1 (1). In the spleen these subsets are not only different in their expression of cell surface molecules but also in their location. $CD8\alpha^+$ DEC-205^+ DCs are located primarily in the T cell zone while $CD8\alpha^-$ $33D1^+$ DCs are located primarily in the red pulp and marginal zones.

The different phenotypes and location of these two subsets are not trivial. Although they share the common ability to take up antigen and present it to T cells, the type of antigen taken up and the type of T cell response generated by the individual subsets is markedly different. $CD8\alpha^+$ DCs specialize in the uptake of apoptotic cells, produce very high levels of

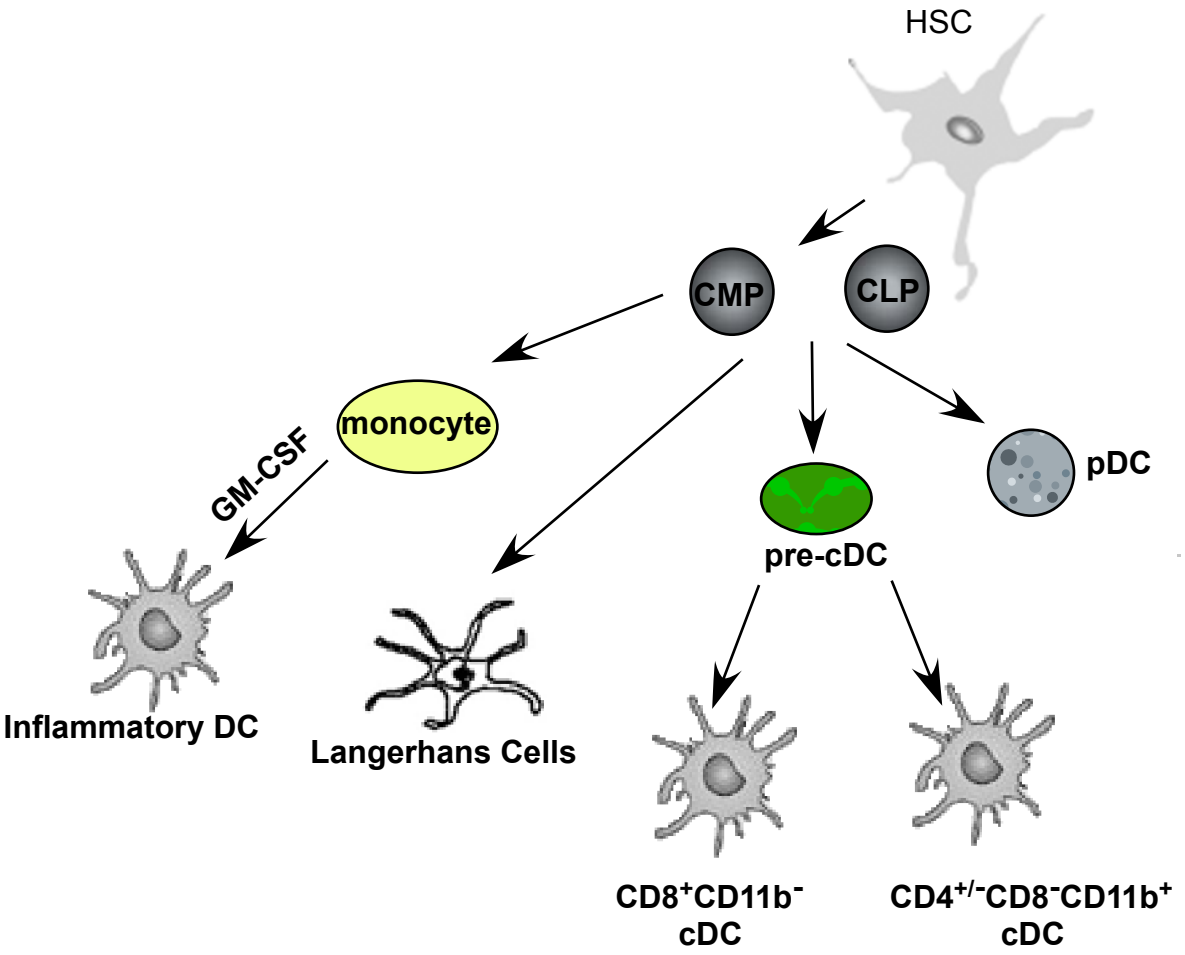


Fig 1.1 Schematic of the basic murine dendritic cell subsets. Hematopoietic precursor cells (HSC) give rise to both common myeloid (CMP) and common lymphoid progenitors (CLP) in the bone marrow. Both types of progenitors have been demonstrated to give rise to both cDC and pDC subtypes.

IL-12p70 upon activation and are most efficient at cross-presentation of exogenous antigens on MHCI for $CD8^+$ T cell activation.

In contrast, $CD8^-$ DCs produce much less IL-12p70, tend to induce T-helper 2 (Th2) type responses and are biased for MHCII presentation and $CD4^+$ T cell activation (2). The capacities of DC subsets to efficiently prime either $CD4^+$ or $CD8^+$ T cells was recently substantiated by microarray studies that identified differential expression of components of MHC I and MHC II processing pathways between the two subsets (1).

In peripheral lymph nodes, using CD8α expression to identify resident DC becomes more difficult. Peripheral lymph nodes contain migratory DC populations in addition to the lymphoid resident cells. Langerhans cells (LC) are the classical paradigm of migratory DC and upon activation, they upregulate CD8α expression. They are generated and reside in the epidermis where they collect environmental antigens and transport them back to the draining lymph nodes. LC migrate at a basal rate even in the absence of infection although migration is increased in response to inflammation and exposure to pathogens.

Plasmacytoid DCs are a very distinct subset of dendritic cells. Like conventional DCs pDC are produced in the bone marrow but unlike cDCs they do not seed the peripheral tissues to migrate to lymphoid organs upon activation. Plasmacytoid DCs circulate in the blood and have been shown to express CD62L similar to naïve lymphocytes (3, 4) and thus use a similar mode of entry into the lymphoid tissues. They reside in lymphoid tissues until they are recruited to sites of infection and inflammation. The pDC are also very restricted in their TLR expression and in the murine model they express only TLR7 and TLR9 (5, 6) thus making them unresponsive to LPS, flagellin, dsRNA and peptidoglycan. However, pDC are

extremely responsive to viral infections and produce copious amounts of type I IFN (10-1000 fold better than their cDC counterparts) (7).

In humans there is also evidence that DCs exist as a heterogenous population although not many studies have been done using isolated tissues. Unlike the mouse, human DCs do not express CD8α so the identification of an equivalent population is difficult (2). The human thymus has at least two DC populations: a $CD11c^{+}CD11b^{-}CD45RO^{lo}$, not expressing myeloid markers, and a $CD11c^{+}CD11b^{+}CD45RO^{hi}$ population that may be the mouse 'myeloid' DC equivalent (8, 9). Humans also have pDCs but unlike mouse pDCs which are $CD11c^{lo,}$ human pDCs are $CD11c^{-}$ but can be identified by their expression of BDCA2 and CD123 (10). Lastly, humans also have a Langerhans cell equivalent that can be identified based on expression of CD1a, langerin and the presence of Birbeck granules.

1.2.2 Dendritic cell activation

Dendritic cells play a central role in pathogen recognition and eradication through activation of the adaptive immune response. On their surface DCs express evolutionarily conserved, invariant receptors that recognize conserved microbial products. Once engaged, these pattern-recognition receptors (PRRs) initiate a signaling cascade that triggers the DC activation and maturation process, transforming them into potent activators of naïve T cells.

The process of DC activation is complex and initiates changes in cell surface molecule expression, phagocytic ability, antigen processing, cell motility and cytokine production. Immature DCs in the periphery shows a high capacity for environmental sampling through macropinocytosis, endocytosis and phagocytosis yet very little of the processed peptide

antigens are present on the surface of the immature DC. Degraded antigens accumulate in MHC class II-rich compartments where the MHCII remains associated with the invariant chain, which can be cleaved by cathepsin S. In immature DCs the activity of cathepsin S is inhibited by high levels of cystatin C. When DCs are activated and undergo maturation, the level of cystatin C is downregulated and the activity of cathepsin S increases allowing for the cleavage of the invariant chain and subsequent surface expression of MHCII-peptide complexes. The peptide-MHC complexes are thought to provide 'signal one' in the three-step model of T cell stimulation by activated DC.

In addition to the presentation of antigen-MHC, DCs undergoing maturation upregulate other surface molecules involved in migration, adhesion and costimulating the adaptive response. The most well known are CD40 and the B7 family members, CD80 and CD86. However, the full range of molecules upregulated by DCs is an active area of investigation and new additions are being discovered all the time. Recent molecules that have come to light are OX40L and 4-1BBL (11) and RANK whose engagement on the DC increases survival, leading to enhanced T cell proliferation (12). Costimulatory molecule engagement provides 'signal two' to T cells.

The third signal activated DCs provide to T cells is through cytokine secretion. DCs activated through their PRRs show specific cytokine secretion patterns that are subset, microenvironment and PRR specific. Exposure to TLR ligands like bacterial LPS or CpG DNA induces a cascade of signaling that results in the secretion of proinflammatory mediators like TNFα, IL-6, type I IFNs and the IL-12 family. These cytokines drive IFNγ production from NK and T cells and skew the immune response towards a Th1 phenotype. Exposure of DCs to TLR2 ligands like zymosan or peptidoglycan can induce the production

of Th1 inhibitory cytokines, such as IL-10 (13). The cytokine pattern generated by activated DCs is crucial in instructing the outcome of the immune response and governs both the Th response and the development of effector cytotoxic T lymphocytes (CTLs). A basic overview of the three signals given to a naïve T cell by an activated DC is shown in figure 1.2

1.2.3 Dendritic cell migration

DCs normally reside in the periphery where they act as immunological sentinels. However one of their most distinct properties is their ability to carry antigenic cargo back to the lymphoid tissue where they liaise with T and B cells. Migratory DCs get to the lymph nodes through the afferent lymphatic vessels but to date the processes and routes taken from the periphery to the lymph node are still not largely understood in the mouse model.

During the process of activation, DCs upregulate the chemokine receptor CCR7, which is required for their ability to traffic to the lymph nodes (14, 15). CCR7 is the 'gate-keeper' for DC entry into the lymph nodes but is not sufficient: other extracellular signals, such as prostaglandin E2 (PGE_2) or nicotinamide adenine dinucleotide (NAD^+), are required to sensitize CCR7 to its ligands, CCL19 and CCL21 (16). In addition to CCR7, integrin mediated adhesion may facilitate the exit of DCs from the lymph vessels and entry into the lymph node where ultimately, the DC must make its way to the T cell areas to initiate the immune response.

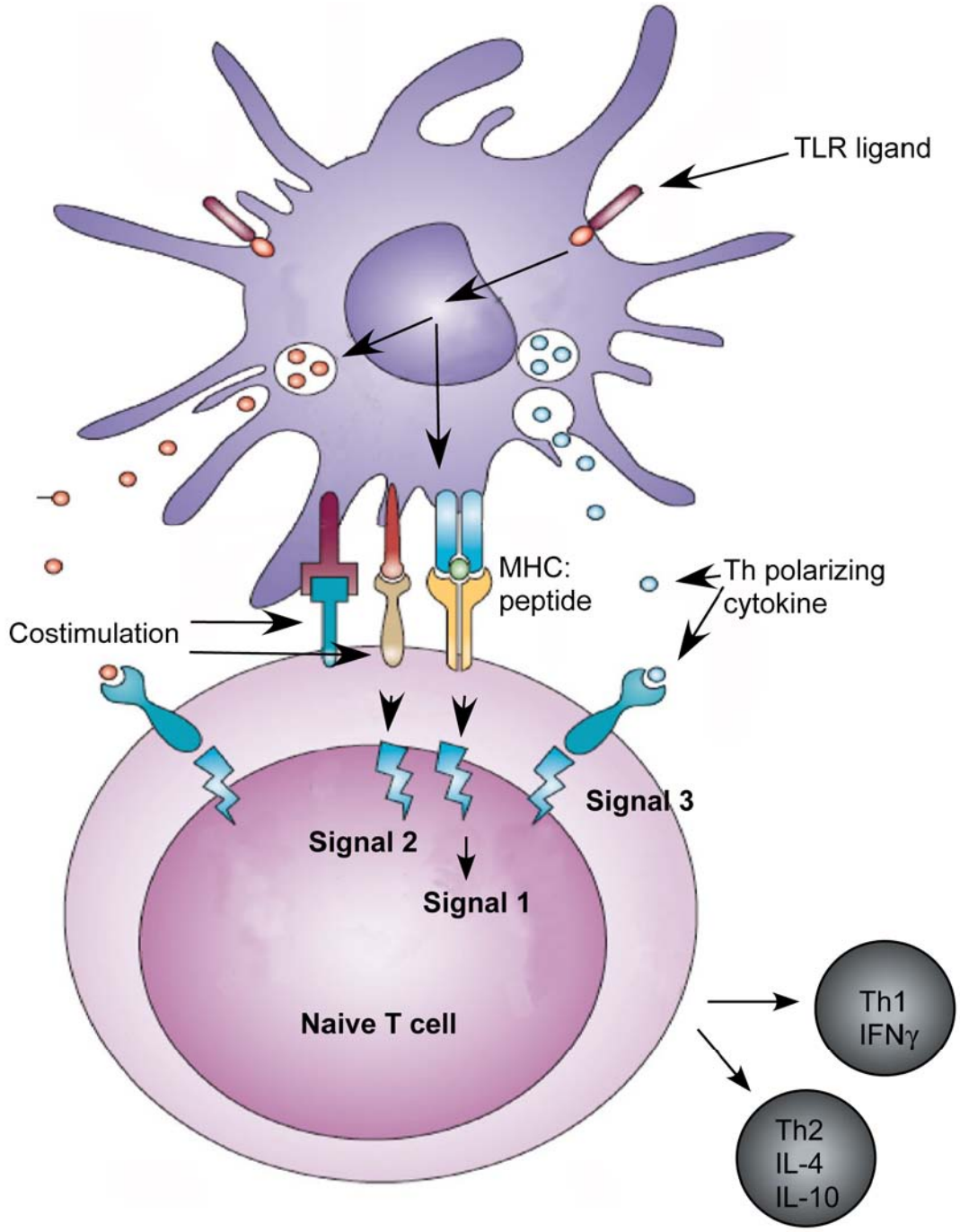


Fig 1.2 Dendritic cell interactions with naive T cells. DCs become activated upon encounter with TLR ligands. Once activated, they upregulate MHC: peptide complexes and costimulatory molecules that deliver signals 1 and 2 to naïve T cells and they also secrete cytokines, providing signal 3, that governs Th-polarization. Adapted from Kapsenburg, M., *Nat Rev Immunol.*, 3:986, 2003.

1.2.4 Dendritic cells in innate immunity: NK-DC cross-talk

Natural killer (NK) cells are a subset of innate immune cells that play an important part in the early response to invading pathogens as well as eradication of autologous cells that are identified as aberrant (for example, lacking or expressing very low levels of MHCI), either through viral infection or malignant transformation. A balance of signals received through both activating and inhibitory receptors governs NK cell activation and effector function. Activation of NK cells results in three different categories of effector function: cytolysis of target cells, cytokine secretion and costimulation of other cells through molecules such as CD40L.

An original study by Fernandez *et al*., (17) demonstrated that contact between NK cells and activated DCs were required to stimulate NK cell cytolytic function and a high level of IFNγ release. Further investigations revealed that NK cell activation by DCs requires both cell-cell contact and a cytokine signal (18). Cytokines produced by activated DCs that have been implicated in NK cell activation include IL-2 (19), IL-12 (20), type I IFN, IL-15 and IL-18 (21). A schematic depicting the interactions between NK cells and DCs is shown in figure 1.3.

Reciprocally, it has been demonstrated that activated NK cells can also induce the maturation of pDC and conventional DCs (22) under low NK:DC ratios (1:5). This interaction is dependent upon cell-cell contact and proceeds via the triggering of the NKp30 receptor on the surface of a subset NK cells resulting in the release of high levels of TNFα and IFNγ that stimulate DC maturation (23). This process is counter-regulated by the killer cell inhibitory receptors and NKG2A. Conversely, under high NK: DC ratios (5:1), immature DCs become

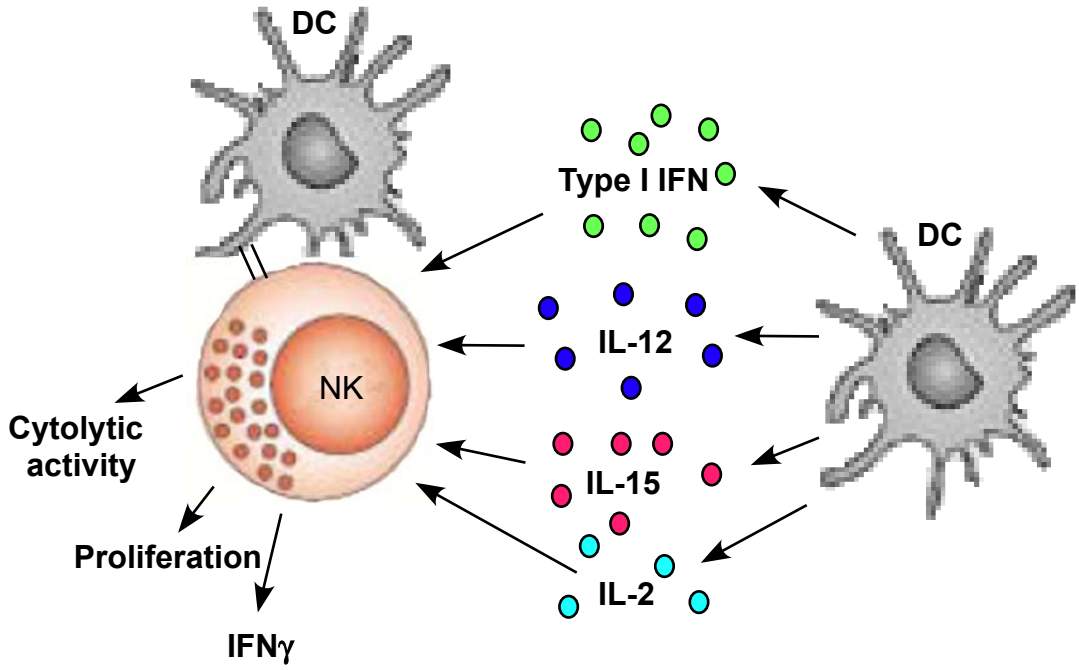


Fig 1.3 Dendritic cell interactions with natural killer cells. Activated DCs secrete several cytokines that can influence NK function such as IL-2, IL-15, IL-12 and type I IFN. An additional cell-cell contact mechanism is also at play to augment cytolytic effector function. Adapted from Moretta, A., *Nat Rev Immunol.,* 2: 959, 2002.

susceptible to NK-mediated cytolysis. The complex relationship between NK cells and DCs is likely an important step in the regulation of DC homeostasis and thus the balance between immunity and tolerance.

1.2.5 Dendritic cells in adaptive immunity

A DC that has encountered a pathogen during its surveillance of the periphery undergoes activation and migration to the secondary lymphoid organs to present its antigenic cargo to naïve T cells. Most classical studies of DC function have revolved around this paradigm while modern *in vivo* imaging techniques have brought new information on the dynamics of this process.

Once activated, a mature DC will express very high levels of antigen-MHC complexes as well as T cell costimulatory molecules such as CD40 and CD80. However, the process of T cell activation is much more complex and involves other factors such as the density of peptide-MHC on the DC surface, the length of contact between the DC and the T cell and even the capture of chemokines like CCL21 on the DC surface and the presence of other T cells subsets like the CD4+CD25+ regulatory T cells (24).

Apart from costimulatory signals generated during T cell-DC contact, cytokine secretion by the DC also plays an important role in shaping the T cell response. Cytokines such as IL-12, IL-23 and the type I IFNs are well-known Th1 polarizing cytokines while IL-10 and TGFβ can inhibit Th1 responses and can result in a Th2 response. The cytokine profile of an activated DC depends both on the subset of DC activated, and on the activation

stimulus itself. For example, pDC produce very high levels of type I IFN inducing Th1 responses (6) while $CD11b^{+}$ DCs are thought to preferentially induce Th2 responses (25, 26). Bacterial LPS induces very high levels of IL-12 that primes IFNγ release from T cells while helminth-derived products inhibit DC-derived IL-12, promote IL-10 production and promote polarization of IL-4 producing T cells (27).

In addition to priming naïve T cells, DCs also play a role in expanding subpopulations of regulatory T cells (Treg) that induce immunological tolerance. The ability of certain DCs to induce T cell tolerance may be subset specific (for example, $CD45RB^{hi}$ DC (28)) or acquired through environmental factors. Some types of tumors have capitalized on this DC function by inducing DCs to produce TGFβ, which promotes Treg proliferation (29). A study by Kretschmer *et al*., (30) also showed that DC expressing very low levels of antigen-MHC complexes to naïve T cells resulted in suboptimal T cell activation and conversion to FoxP3 expressing regulatory cells. In addition to the production of soluble factors such as IL-10 and TGFβ, expression of inhibitory cell surface receptors on the DC like ILT3 and ILT4 (31) or B7-H4 have also been shown to render the DC immunosuppressive (32) by conveying T cell inhibitory signals as opposed to costimulatory ones.

Much of the role of DCs in adaptive immunity has centered on T-DC interactions. However, DCs are also important for the antibody response (33). Although this was initially believed to occur indirectly as a result of helper T cell licensing by DCs, DCs have since been demonstrated to interact directly with B cells to initiate their proliferation and secretion of both IgM and IgG (34). DCs can also transfer antigen to naïve B cells and give CD40-dependent survival signals (35).

1.2.6 Dendritic cell activation of T cells versus immunosuppression

One of the most unique features of DCs is their ability to induce either immunological activation or tolerance. Tolerance can be broadly divided into two categories: central and peripheral. It has long been believed that DCs play an important role in the deletion of autoreactive T cells in the thymus by presentation of self-antigens (36). However, central tolerance has limitations and some self-reactive cells escape deletion and exit to the periphery. Mechanisms of peripheral tolerance are required to prevent activation of these cells and resultant damage to self.

The original hypothesis regarding peripheral tolerance induction states that immature DCs are responsible for induction of tolerance due to their inefficient presentation of MHC: peptide complexes in conjunction with their low levels of costimulatory molecules (37). Evidence derived from *in vivo* antigen targeting to steady-state DCs through endocytic receptors supports this theory (38, 39). Immature DCs have been cited to induce peripheral tolerance through T cell deletion; induction of T cell anergy (40) and through induction of Treg cells (41). However, the discovery of tolerance induction in $CD4^+$ T cells by phenotypically mature DCs (42, 43) and the discovery of partially mature DCs in secondary lymphoid organs in the steady-state has called the bimodal theory of tolerance into question. It is now becoming accepted that it is the maturation stimulus in conjunction with environmental and intrinsic properties of the DC itself that govern the switch between activation and tolerance.

Recent work by Wakkach *et al*. (28) identified a novel regulatory DC subset capable of inducing IL-10 producing regulatory T cells. Interestingly, this subset was marked by its

expression of high levels of CD45RB. Another study found that DCs are capable of expanding FoxP3^{+}CD25^{hi} Treg cells and, surprisingly, the more mature DCs (CD86^{hi}) were even more efficient (44) than the immature cells. These are both examples of where the innate capabilities of the DCs themselves function to elicit tolerance.

Environmental factors that slant DCs towards a more tolerogenic function include cell-cell communication and cytokine environment. In tissues, stromal cells are an important factor in creating the microenvironment. Zhang *et al*., conducted a study where DCs were differentiated on mouse embryonic splenic stromal cells (45). TGFβ and stromal cell contact drove the DCs to acquire a tolerogenic phenotype resulting in the suppression of T cell proliferation through secretion of nitric oxide. Soluble mediators secreted by both the DCs themselves and by neighboring cell types are clear examples of situations where environmental signals can skew DC effector function towards tolerance.

1.3 Dendritic Cells and Toll-like Receptors

1.3.1 Pathogen recognition and Toll-like receptors

Dendritic cells have many receptors whose function is to recognize conserved microbial products, often called 'pathogen-associated molecular patterns' or PAMPs. These are features vitally connected to the function of the microorganism such that any evolutionary change would have resulted in dire consequences for the pathogen. The model PAMP is LPS, an essential cell wall-component of most Gram negative bacteria. Others

include peptidoglycan from Gram positive bacteria, unmethylated CpG DNA that is found in bacterial cells, and viral products like double-stranded RNA.

The receptors used by innate immune cells to detect PAMPs are germ-line encoded; invariant receptors often termed 'pattern-recognition receptors' or PRRs (46). There are many different classes of PRRs expressed by DCs including the C-type lectin receptors, mannose receptors and the best studied, the Toll-like receptors (TLRs). In 1996 it was demonstrated that the Toll proteins in Drosophila are necessary for the flies to mount an effective immune response to the fungus *Aspergillus fumigatus* (47). In 1997, Medzhitov and Janeway identified a mammalian homologue (TLR4) that was found to induce genes involved in the inflammatory response (48). Since then up to twelve TLRs have been identified in the mouse genome and ten human TLRs have been cloned.

1.3.2 Toll-like receptor structure

As conserved PRRs, TLR family members share a similar structure. They are large type I transmembrane glycoproteins that are either found on the cell surface (for detection of bacterial and fungal invaders) or located in intracellular compartments (for detection of viruses and non-self nucleic acids). The cytoplasmic domains of the TLRs have a high degree of homology to the interleukin-1 receptor (IL-1R) and thus they are also part of the IL-1R superfamily although their extracellular domains differ considerably. Examples of the TLRs and their ligands are depicted in figure 1.4.

The extracellular domains of the TLRs contain 19-25 blocks of a 24-29 amino-acid motif that is rich in leucines and other hydrophobic amino acids (leucine-rich repeats or

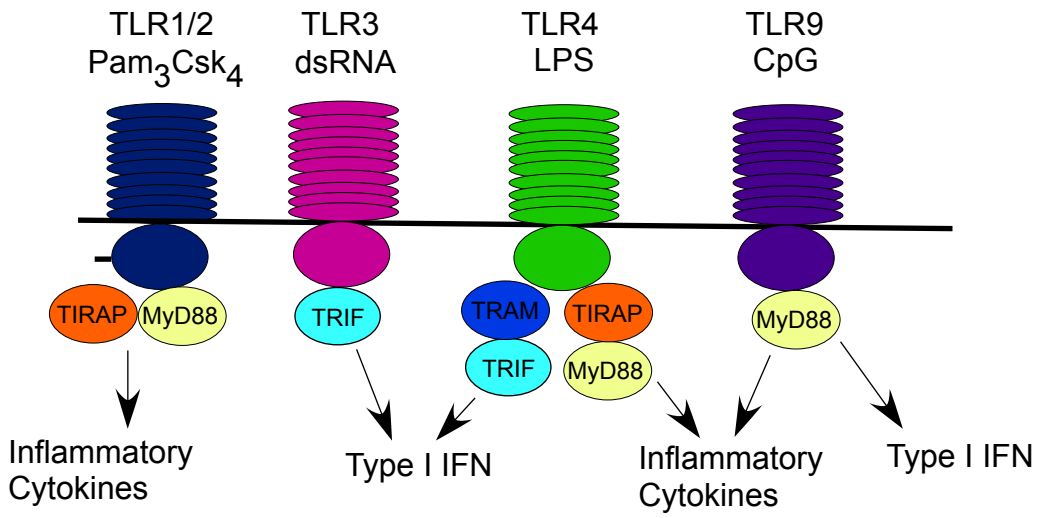


Fig 1.4 Toll-like receptors and their ligands. Different TLRs recognize different pathogen-specific molecules and preferentially utilize different adaptor molecules leading to different outcomes. MyD88 is one adaptor that is common to all TLRs except TLR3.

LRRs) (49) capped with a cysteine-rich structure at both the carboxyl and N-termini (50). The LRRs are thought to fold into a horseshoe shaped, solenoidal structure with the hydrophobic amino acids on the concave surface, which is thought to be the area involved in ligand binding (51, 52). A rendering of the basic TLR structure is shown in figure 1.5 and is based on the crystal structure of TLR3. The case of TLR4 is an exception to this simplified structure since TLR4 can only bind to LPS when it is complexed with MD-2. In this instance, he concave surface accommodates both molecules (53).

Each TLR has a different, non-structurally related, specific ligand but a similar extracellular structure and it is thought that ligand specificity is generated in part by the different cellular locations of the TLRs (for example, TLRs 1, 2, 4, 5, and 6 are on the cell surface while TLRs 3,7,8 and 9 are located intracellularly in endosomes), interactions of side-chains of the variable amino acids in the LRRs with the ligands (50) and their ability to form heterodimers as is the case for TLRs 2/6 and TLRs 1/2.

The cytoplasmic domain of the TLRs and the IL-1R share a conserved region of about 200 amino acids known as the Toll/IL-1R domain (TIR) (54). All TIR domains have three conserved regions, or boxes, necessary for signaling and outside of these boxes only share a 20-30% sequence homology. Signaling through the TLRs is thought to occur by ligand-induced dimerization (55) that brings together TIR domains on each partner and creates a place for other TIR-domain containing adaptor molecules to interact, linking them to intracellular signaling proteins.

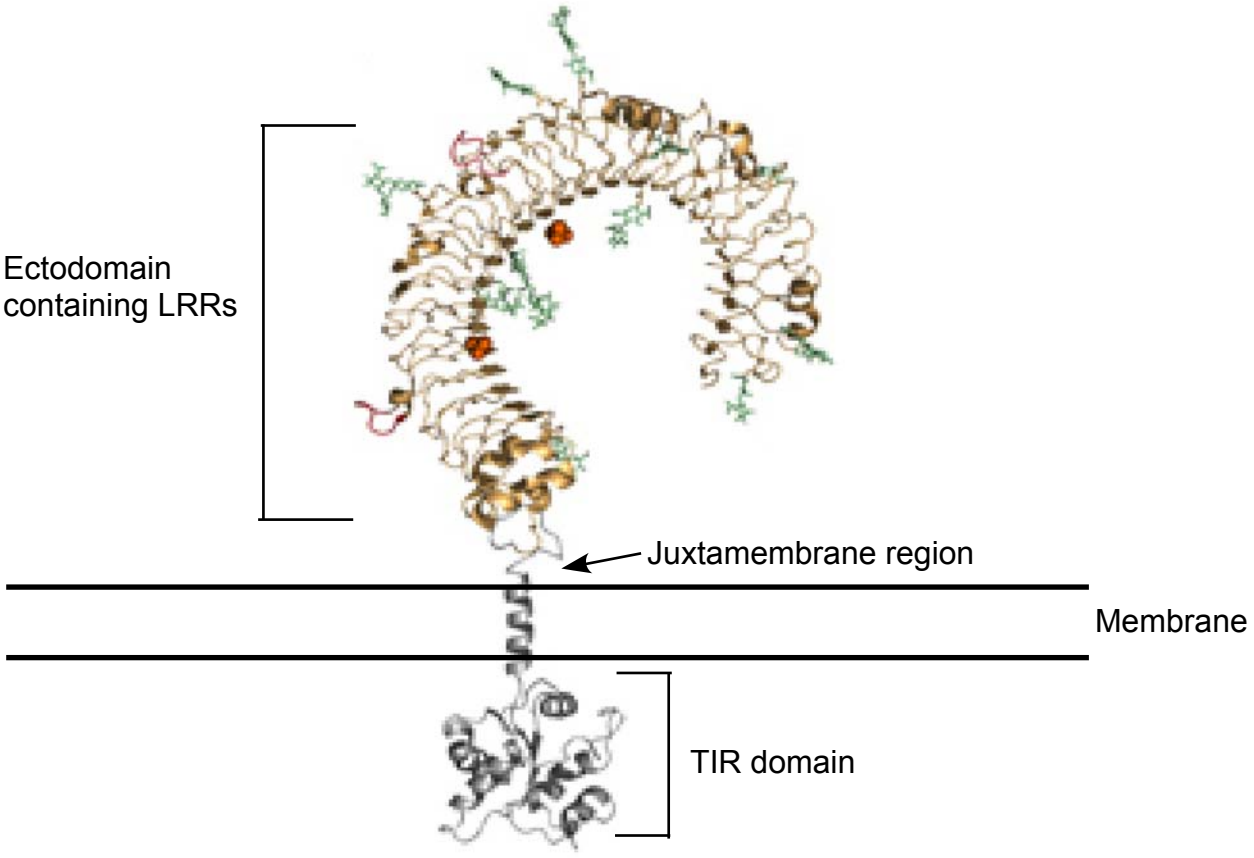


Fig 1.5 Toll-like receptor structure. The extracellular domain of the TLR folds into a horseshoe-shaped structure where ligand binding occurs at the hydrophobic concave surface. The intracellular domain contains the TIR-domain that mediates interactions with adaptor proteins like MyD88. Adapted from Bell *et al*., *PNAS* 102:10977, 2005.

1.3.3 Toll-like receptor signaling: the MyD88-dependent pathway

Following ligand binding to the TLR, a specific series of adaptor molecules gets recruited to the cytoplasmic domain of the TLR via TIR-TIR interactions. The first adaptor to be recognized as essential for IL-1R/TLR signaling was myeloid-differentiation factor 88 (MyD88) so named because of its rapid induction during IL-6-mediated differentiation of M1 myeloleukemic cells into macrophages. The cloning of MyD88 revealed its function as an adaptor protein that links IRAK4 to the IL-1R signaling complex (56-58) and then later, to human TLR4 (59). The necessity of MyD88 to TLR signaling was demonstrated by knockout studies showing that although the mice appeared normal, their cells lose the ability to produce proinflammatory cytokines in response to several different TLR ligands (60, 61) and thus the mice are resistant to endotoxic shock (62). The only TLR pathway identified that does not use MyD88 for signaling is TLR3.

The second TLR adaptor to be identified was Mal/TIRAP (63, 64). Studies of Mal knockout mice linked Mal to MyD88. The $Mal^{-/-}$ mice also showed decreased proinflammatory cytokine secretion but only in response to stimulation by TLRs 2 and 4 (65, 66). Like the MyD88 knockout mice, cells deficient in Mal also showed a delayed activation of NF-κB and mitogen activated protein kinases (MAPKs) following TLR4 stimulation but they also showed a complete impairment of p38 and NF-κB activation following TLR2 stimulation. It is now known that Mal binds phosphatidylinositol-4,5-bisphosphate in the plasma membrane (67) where it can reside near the TLRs and act as a bridge for MyD88 recruitment during TLR2 and TLR4 signaling. Unlike MyD88, Mal is also able to associate with TRAF6 (tumor necrosis factor receptor activated factor) and thus recruit TRAF6 to the

TLR2 and TLR4 signaling complexes (68). It can also interact with Bruton's tyrosine kinase (Btk) (69), a Lyn substrate in B cells. Differential use of Mal between the TLRs was the first evidence that recruitment of specific adaptor molecules might be a key to TLR signaling specificity.

Immediately downstream of MyD88 are the IL-1R associated kinases (IRAKs). IRAK4 activates IRAK1 that subsequently interacts with the ubiquitin E3 ligase, TRAF6. Ubiquitination of TRAF6, by itself, then recruits a complex containing transforming growth factor-β activated kinase (TAK1) and its associated proteins. TAK1 is the central kinase in the complex that can mediate phosphorylation of IKK and can mediate IκB phosphorylation, leading to its ubiquitination and degradation. This frees NF-κB to translocate to the nucleus and mediate transcription of its target genes. TAK1 is also responsible for activating MKK6 (mitogen activated protein kinase kinase 6) that leads to the activation of p38 and JNK and subsequent activation of AP-1 (70).

The activation of NF-κB and AP-1 are the most well studied transcription factors activated by TLR signaling but not the only ones. MyD88 also leads to the activation of several interferon-response factor (IRF) family members whose contribution to TLR signaling is not as well understood. One of the major IRFs downstream of MyD88 is IRF5. IRF5 interacts directly with the MyD88-TRAF6 complex that leads to its activation, nuclear translocation and transcription of proinflammatory cytokines. Mice deficient in IRF5 had a severe impairment in proinflammatory cytokine production but not in type I IFN production (71). MyD88 also acts upstream of IRF1, which leads to the synthesis of IFN-β inducible nitric oxide synthase (iNOS) and IL-12p35 (72) and. in pDCs, MyD88 is required for IRF7 activation leading to IFNα production downstream of TLRs 7, 8 and 9 (73).

1.3.4 Toll-like receptor signaling: the MyD88-independent pathway

The delayed activation of MAPKs and NF-κB following TLR4 ligation in MyD88$^{-/-}$ mice suggested the existence of another pathway. Database searches for TIR-domain containing proteins (66) and yeast two-hybrid screens (74) identified the essential adaptor protein for the MyD88-independent signaling pathway as Trif/TICAM-1. The Trif knockout mice are unable to produce IFNβ or activate IRF3 downstream of TLR3 or TLR4 ligation. They also show hampered proinflammatory cytokine production following activation through TLR4 (75). The double knockout of MyD88/Trif in mice abolished the activation of all MAPKs and NF-κB following TLR4 ligation, revealing its role as the mediator of the delayed signaling observed in the MyD88 single-knockout mice (76).

In MyD88-independent TLR3 and TLR4 signaling, Trif complexes with the IKK-like kinase, TBK1, IKKε and IRF3. This leads to the phosphorylation and activation of IRF3 and results in type I-IFN gene transcription. For TLR4 signaling, the adaptor TRAM is required to recruit Trif to TLR4 for IFNβ production to occur but there was no effect on TLR3-mediated IFNβ production in TRAM knockout cells (77, 78). A recent study by Kagan *et al*. (79) has solved the mystery of how TLR4 can bind both MyD88 and Trif that compete for binding on the TIR domain. Kagan was able to show that at the surface, the TLR4-LPS complex signals through MyD88 but then once TLR4 is endocytosed, a second wave of Trif signaling is initiated from the endosome. A summary of the TLR signaling pathways is presented in figure 1.6.

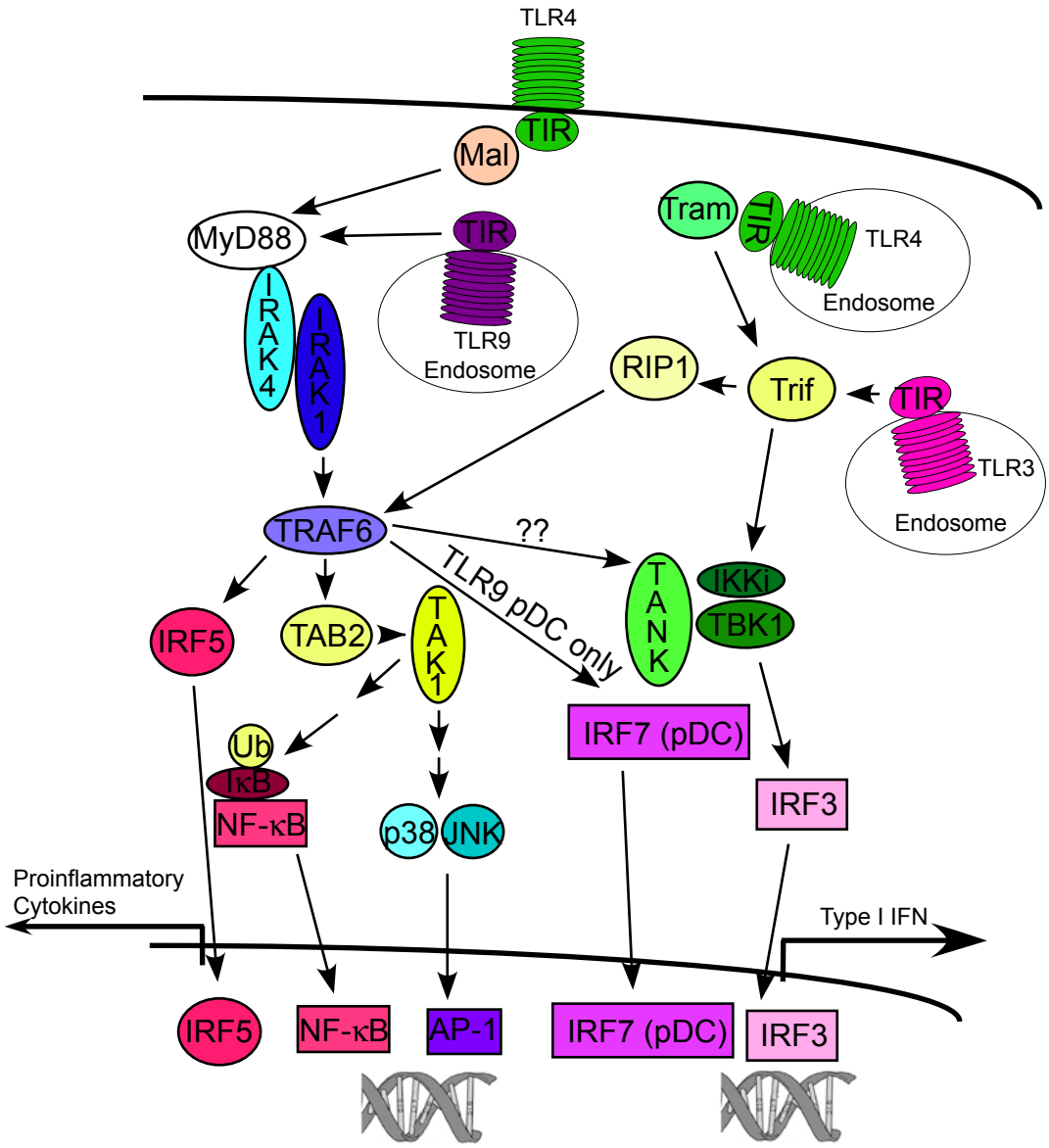


Fig 1.6 Basic diagram of TLR signaling pathways.

1.4 CD45

CD45 is a transmembrane protein tyrosine phosphatase that is expressed on the surface of all nucleated cells in the hematopoietic system, including T cells, B cells, macrophages and DCs. The importance of CD45 is highlighted by the observation that CD45-deficient mice have major defects in T cell development and the discovery that humans and mice lacking CD45 are severely immunocompromised (80).

The best-characterized substrates for CD45 are members of the Src-family of protein tyrosine kinases (SFKs). SFKs play key roles in signal transduction through the T and B cell receptors and can also contribute to growth factor, cytokine and integrin signaling. More recent evidence has also implicated CD45 in regulating Janus kinase (Jak) function (81). Jaks have a central role in transducing signals through cytokine receptors. Thus CD45 substrates participate in a diverse array of immunological functions putting CD45 in a central position to regulate the immune response.

1.4.1 The CD45 extracellular domain

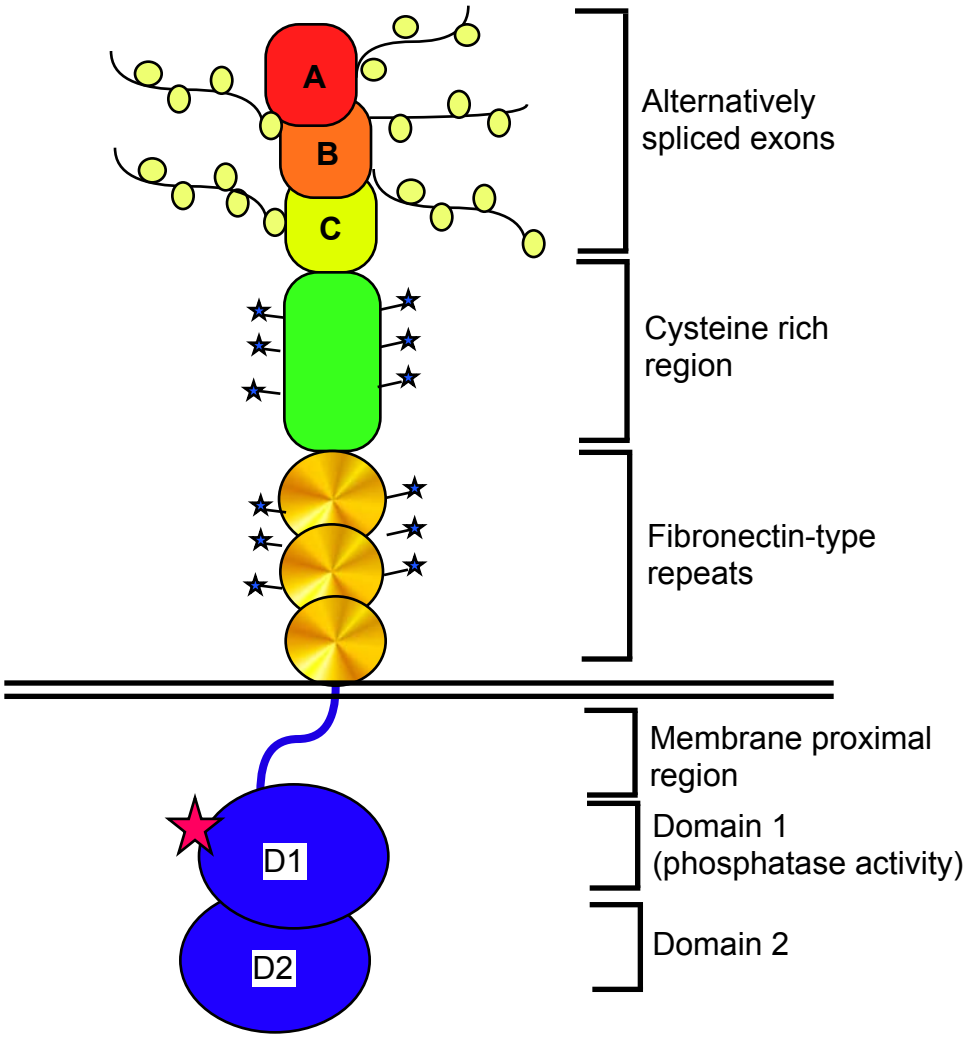
CD45 is a large glycoprotein that is expressed abundantly on the cell surface, often comprising from 5-10% of surface membrane proteins. Multiple isoforms of the extracellular domain of CD45 can be expressed through alternative splicing of at least three exons (designated A, B, and C). The largest isoform, CD45RABC contains all three exons while the smallest isoform, CD45RO, contains none of them. Isoform expression is tightly regulated in

different cell types and at different cell stages but their individual contributions to CD45 function are poorly defined (80).

Cell specific isoform expression has been well characterized in T cells (82). Interestingly, $CD4^+$ T cells with regulatory properties express low levels of the CD45RB isoform (83), (84). An antibody to CD45RB is able to suppress graft rejection (85) (86), prevent experimental autoimmune encephalomyelitis (87) and prevent the onset of diabetes in NOD mice (88). Recent evidence has also linked CD45RB expression on DCs with the ability to induce tolerance. Research has demonstrated the existence of a subset of DCs that expresses high levels of CD45RB and that can mediate tolerance through the induction of regulatory T cells (28). The contribution of CD45 to the tolerogenic properties of these cells has not been established. The structure of CD45 is shown in figure 1.7.

1.4.2 The role of CD45 in T cell development and signaling

In T cells, CD45 is required for signaling through the T-cell antigen receptor (TCR). Three groups have generated CD45 knockout mice independently by targeting exons 6, 9 and 12 (89-91). All of the mice have severe and profound defects in T cell development. In $CD45^{ex9/ex9}$ and $CD45^{ex12/ex12}$ mice, T cell development is partially blocked at the $CD25^+CD44^-$ (DN3) stage indicating a defect in pre-TCR signaling. The result is a five-fold increase in the numbers of DN3 cells and a two-fold reduction in the numbers of double-positive thymocytes (90) (91). In all of the CD45 knockout mice, signaling through the mature TCR is defective and cell development to the single positive stage is severely



1.7 Structure of CD45. The extracellular region has three alternatively spliced exons (A, B and C) that have many sites for O-linked glycosylation. The rest of the extracellular domain consists of a cysteine-rich region and three fibronectin-type repeats that are heavily N-glycosylated. The intracellular region has two tandem phosphatase domains (D1 and D2) but only D1 is enzymatically active.

impaired (89-91). Only 5-10% of the normal numbers of T cells are released into the periphery.

Studies with $CD45^{-/-}$ TCR transgenic mice and fetal-thymic organ culture show that $CD45^{-/-}$ cells can undergo positive selection but that they require strong signals in order for development to proceed (90, 91). This hypothesis is supported by research showing that 55% of the peripheral T cells in $CD45^{-/-}$ mice are self-reactive (92) but because these cells are hyporesponsive to TCR stimulation, no autoimmunity is observed. Taken together, these results demonstrate that CD45 alters the signaling threshold in T cells.

The failure of $CD45^{-/-}$ mice to develop mature T cells is due to dysregulation of Lck, the SFK essential for transduction of signals through the TCR. The SFKs Lck and possibly Fyn are required for phosphorylation of immunoreceptor tyrosine-based activation motifs on CD3ε and ζ chains (80). This is the earliest event following TCR ligation. Lck and, to a lesser extent, Fyn are the main substrates for CD45 in T cells. SFK activity is controlled by phosphorylation of two key tyrosine residues. Autophosphorylation of a site within the kinase activation loop is necessary for kinase activity and this is balanced by phosphorylation of a C-terminal tyrosine residue. Phosphorylation of the C-terminal tyrosine causes an intramolecular interaction to occur with the kinase's own Src-homology 2 (SH2) domain, causing a conformational change that prevents kinase activity. CD45 can dephosphorylate both key tyrosine residues in SFKs, suggesting that it is both a positive and a negative regulator of SFK activity in T cells (80).

Other possible substrates of CD45 are the Jaks. In $CD45^{-/-}$ Jurkat T cells, cytokine stimulation resulted in hyperphosphorylation of Jak1. These results were also observed after

cytokine stimulation of $CD45^{-/-}$ thymocytes, B cells, macrophages and mast cells (81), demonstrating a role for CD45 as a negative regulator of Jaks.

1.4.3 CD45 in B cell development and function

As in T cells, CD45 is an important modulator of signaling thresholds through the B cell receptor (BCR). In $CD45^{-/-}$ mice, B cell development is largely normal until the final maturation stage. Despite having a two-fold increase in the total number of B cells, the total number of mature B cells, $IgM^{hi}IgD^{low}$, is decreased (91). CD45-deficient B cells are hyporesponsive to IgM and IgD stimulus. As in T cells, the signaling threshold in $CD45^{-/-}$ B cells is higher. In the hen-egg lysozyme (HEL) transgenic system, the HEL autoantigen normally causes negative selection of B cells. On a $CD45^{-/-}$ background, HEL-binding B cells are positively selected (93).

Following ligation of the BCR, the SFK Lyn is recruited to phosphorylate the BCR ITAM (immunoreceptor tyrosine based activation motif). Although the SFKs Fyn and Blk are also present in B cells, Lyn has a unique role in positive and negative regulation of BCR signaling (80). Lyn is also required for phosphorylation of the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) on inhibitory receptors, like FcγRIIB, that down regulate immune responses (80). As with Lck and Fyn, CD45 can dephosphorylate Lyn at both its positive and negative regulatory sites (94).

Other evidence for CD45 function in B cells comes from *in vivo* studies on transgenic mice expressing a point mutation in the juxtamembrane wedge region of CD45 (95). Previously, these mice were shown to develop a lymphoproliferative disorder with lupus-like

autoantibody production leading to the deposition of immune complexes in the kidneys resulting in renal failure (96). The study by Hermiston *et al*., (95) demonstrated that this disorder is mediated by hyperresponsive B cells in which Lyn was hyperphosphorylated on the negative regulatory C-terminal tyrosine. Additionally, introduction of the wedge mutant in mice also altered B cell development, favoring the development of natural antibody producing B1 cells at the expense of conventional B2 B cell. This phenotype is opposite to that observed in the $CD45^{-/-}$ mice (90, 93, 95).

1.4.4 CD45 in macrophage and mast cell function

Unlike the lymphoid compartment, cells of the myeloid lineage develop normally in the absence of CD45. No defects in macrophage development have been reported and one group has observed an increase in $CD45^{-/-}$ bone marrow-derived mast cell proliferation in response to IL-3 (81). In macrophages, the best-characterized function of CD45 is during integrin-mediated adhesion. CD45 deficient macrophages adhere to and spread rapidly on tissue culture plastic dishes, a process regulated by β2 integrins (97). Although $CD45^{-/-}$ macrophages have increased kinetics of adherence, this signal is not sustained and after 48hrs the $CD45^{-/-}$ macrophages detach while the $CD45^{+/+}$ cells remain attached. The SFKs Hck and Lyn are hyperphosphorylated and have enhanced activity in the CD45-deficient macrophages suggesting that these SFKs are the main CD45 substrates in macrophages.

In mast cells, CD45 is required for degranulation in response to IgE-mediated cross-linking of FcεRI (98) (81). CD45 knockout bone marrow-derived mast cells do not degranulate upon IgE cross-linking and $CD45^{-/-}$ mice are resistant to IgE mediated

anaphylaxis (98). The SFK phosphorylation state in these cells has not been reported, however; Lyn is a known regulator of FcεRI signaling (99). A paper by Tolar *et al.,* has shown that expression of a constitutively active form of Lyn in RBL-2H3 cells causes a 50% decrease in degranulation (100). Since Lyn activity in $CD45^{-/-}$ macrophages is increased, the results in the RBL-2H3 cells are likely to represent the situation in $CD45^{-/-}$ mast cells. The decrease in receptor signaling can be reconciled with an overactive Lyn since another target for Lyn in mast cells is the inhibitory receptor FcRγIIB (101). It is likely that an increase in the phosphorylation of ITIMs on this receptor, by an activated Lyn, causes inhibition in mast cell response.

In summary, a significant amount of data has been collected regarding the role of CD45 in lymphoid cells but significantly less is understood about its function in other leukocytes such as DCs. CD45 is a central regulator of many pathways that are important to immunological function. An efficient immune response requires that the DCs recognize a danger signal, upregulate antigen presentation and co-stimulatory molecules and migrate to the lymph nodes. In the lymph nodes the DCs must form a tight contact with the T cells. Stimulated DCs and T cells engage in cytokine-mediated 'cross-talk' that drives the nature and quality of the immune response. CD45 and its substrates can mediate: cell development, receptor signaling thresholds, cell adhesion, FcR signaling and Jak-mediated cytokine responses. The goal of this thesis is to determine the contribution of CD45 to some of these processes in DCs.

1.5 Thesis objectives

It is clear that there is a large body of literature devoted to determining roles for CD45 in many immunological cell types and most of that work has focused on how CD45 regulates SFKs to exert its effects. SFKs are present in DCs, as are Jaks, but at the time this work was initiated, little was known about what processes these signaling molecules affect in DCs and what role CD45 plays in these cells. CD45 is a leukocyte specific protein tyrosine phosphatase. CD45 is very highly expressed on the surface of myeloid cells. In lymphoid cells CD45 is central to signaling through the antigen receptor and without CD45 these cells do not develop properly however, myeloid cells like macrophages do develop in $CD45^{-/-}$ mice. Thus, the first aim of this thesis was to determine if the lack of CD45 would hinder or alter the *in vivo* and *in vitro* development and function of dendritic cells. DCs that have developed properly express surface costimulatory molecules that can be upregulated upon TLR engagement as well as being capable of the production of proinflammatory cytokines. These functions are critical to the induction of an immune response.

The second major aim of this thesis was to try to determine where CD45 might exert its effects on TLR signaling. To achieve this, SFK family members present in DC were profiled for their phosphorylation state and substrates of the SFKs that have identified roles in TLR signaling were also examined. The activation of signaling molecules central to TLR activation was also examined. The final objective of this work was to look for a functional consequence of the lack of CD45 in DCs on the outcome of an immune response.

Chapter 2: Materials and methods

2.1 Materials

2.1.1 Mice

C57BL/6 (hereafter referred to as wild type) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained in the Wesbrook Animal Unit at UBC through homozygous matings. $CD45^{-/-}$ mice with a targeted disruption in exon 9 (90) were initially purchased from Jackson Laboratories and backcrossed onto the C57Bl/6 background three more times to generate a ninth generation backcross (hereafter referred to as $CD45^{-/-}$) that were then maintained by homozygous matings. TCR transgenic mice specific for $OVA_{323-339}$ peptide (OTII) (102) were also obtained from Jackson Laboratories and maintained by homozygous matings. RAG2 knockout mice (103) (obtained from Dr.Teh's lab, UBC) were crossed onto the $CD45^{-/-}$ strain to yield $CD45^{-/-}RAG2^{-/-}$ double-deficient mice. Mice were housed and maintained according to the Canadian Council of Animal Care guidelines and all experiments were conducted in accordance with the guidelines set out by the Animal Care Committee at the University of British Columbia. Animals used for experiments were generally between 8 and 16 weeks of age, and were age and sex matched within each individual experiment.

2.1.2 Cell isolation and culture

In vitro activation of splenic DCs was accomplished by digesting minced spleens from wild type or $CD45^{-/-}$ mice in 1mg/ml collagenase D (Roche Diagnostics, Laval, QC) at room temperature for 30 minutes in PBS supplemented with 1% FCS. Digestion was stopped by the addition of EDTA to a final concentration of 20mM with a 5 minute incubation. Digested spleens were passed through a wire mesh to generate a single cell suspension and the red blood cells were then lysed for 5 minutes with a 0.84% NH_4Cl solution. Splenocytes were washed and plated at $1x10^6$ cells/ml in complete media comprised of RPMI 1640 (Invitrogen, Burlington, ON) supplemented with 10% FCS, 2mM L-glutamine, 2mM sodium pyruvate, 1X non-essential amino acids (Invitrogen, Burlington, ON), 20mM HEPES and 50µM 2-mercaptoethanol and cultured at 37°C in 5% CO_2 .

Bone marrow derived dendritic cells (BMDCs) were obtained according to the protocol of Lutz et al. (104). Basically, bone marrow was flushed from the femurs and tibiae of mice into a solution of HBSS with 2% FCS and 5mM EDTA. Cells collected were washed, the red blood cells lysed and then they were counted for plating at a density of $2x10^5$ cells/ml in complete media (as for splenocyte culture) with the addition of 4% (v/v) supernatant from the GM-CSF secreting cell line, J558L (105). On day 3 or 4, an equal volume of media with fresh GM-CSF was added to the plates. On day 6, half of the culture was removed and replaced with fresh media and GM-CSF. Cells were harvested for use on day 7 or day 8.

T cells for use in stimulation experiments were obtained from the spleen and peripheral lymph nodes of OTII mice. At least 2 mice were harvested per isolation. Single

cell suspensions were made and the red blood cells were lysed prior to purification of CD4+ T cells by MACS negative selection (Miltenyi Biotech, Auburn, CA) according to the manufacturer's protocol. Purity, as assessed by flow cytometry, was generally around 85%. Freshly isolated T cells were then cocultured with BMDC in the presence of peptide (synthesized at UBC NAPS facility) in complete media at 37°C in 5% CO_2.

Natural killer cells were isolated from splenocyte suspensions from wild type mice. At least two spleens were used per experiment. Isolation was similar to T cells except that NK cells were positively selected using the MACS positive selection kit based on the expression of DX5. Isolated NK cells ($2x10^5$) were immediately cocultured with BMDC ($1x10^5$) in complete media with or without 1μg/ml of LPS (Invivogen or Sigma) at 37°C in 5% CO_2.

2.1.3 Antibodies

Antibodies specific for mouse CD11c (N418), CD40 (HM40-3), CD80 (16-10A1), CD86 (GL1), MHCII I-A/I-E (M5/114.15.2), CD4 (RM40-3), CD11b (M1/70), DX5, CD8α (53-6.7), IL12p40 (C17.8), TNFα (MP6-XT22), IFNγ (XMG1.2), TLR4 (MTS510) and Gr-1 (RB6-8C5) were all purchased from eBioscience (San Diego, CA) conjugated to either FITC, PE, PE-Cy5, APC, PE-Cy7 or APC Cy7. Plasmacytoid dendritic cells were detected with PE-labeled mPDCA-1 antibody from Miltenyi Biotec (Auburn, CA). The pan-CD45 antibody used was I3/2-Alexa488 (purified from tissue culture supernatant and coupled to Alexa488 using a kit from Molecular Probes). Antibodies to CD45 isoforms were CD45RA-Alexa488 (14.8)(purified from tissue culture supernatant and coupled), B220-FITC (RA3-6B2)(eBioscience), CD45RB-Alexa488 (MB23G2) (purified from tissue culture supernatant

and coupled) and CD45RC-FITC (GL24) (eBioscience). Dendritic cell FcRs were blocked using anti-CD16/32 (2.4G2, ATCC, Manassas, VA) tissue culture supernatant prior to antibody labeling. For signaling studies, antibodies specific for Jak1, Jak2, Lyn, Hck, Fyn (sc-16), Fgr, DOK2, Cbl and NF-κB p65 were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Src as well as the phospho-specific antibodies to Erk, p38, Akt (Ser473), Jnk, phospho-Src416 and phospho Lyn507 were obtained from Cell Signaling Technology (Pickering, Ontario), as was the anti-phosphotyrosine mAb (4G10). Anti-SHIP sera was a gift from Gerry Krystal (Terry Fox Laboratories, Vancouver, BC).

2.2 Methods

2.2.1 Flow cytometry

Surface antigens were detected by incubation with primary antibody in a 96-well plate subsequent to FcR blocking in 100ul of tissue culture supernatant from the 2.4G2 anti-CD16/CD32 hybridoma. Antibody incubation steps were done for 20 minutes on ice in FACS buffer (PBS/2% FCS/5mM EDTA). After antibody incubations, cells were washed in FACS buffer and, where appropriate, dead cells were detected by incubation for 5 minutes in a solution of 7-amino-actinomycin D (7-AAD). Dead and auto-fluorescent cells were excluded from analysis. Between $2x10^5$ and $1x10^6$ cells were labeled per well and where possible, a minimum of 20000 counts were collected for analysis. Negative staining was determined in some cases by inclusion of appropriate isotype control antibodies and in other cases a fluorescence-minus-one control was used. Labeled cells were analyzed either on a

FACscan, FACSCalibur or LSRII flow cytometer (Beckton Dickson, Mississauga, ON) and data was analyzed using FlowJo (TreeStar, Ashland, OR).

For intracellular cytokine staining, the initial surface labeling was performed as described above with the exception of the addition of the 7-AAD. After primary antibody incubation, the cells were washed and then incubated in fixation/permeabilization buffer containing 2% paraformaldehyde/0.5% saponin in PBS for 20-25 minutes on ice. Cells were washed in permeabilization/wash buffer containing PBS/2% FCS/0.5% saponin and then incubated with antibodies to intracellular cytokines (or appropriate isotype control anibodies) in the same buffer for 30 minutes on ice. The labeled cells were then washed in permeabilization/wash buffer twice and then resuspended in FACS buffer for flow cytometry.

2.2.2 Partial purification of splenic dendritic cells

Spleens from mice were first perfused with 1ml of a collagenase D (Roche Diagnostics, Laval QC) solution (1mg/ml collagenase D, 2% FCS in PBS), minced into another 1.5ml of the same solution and left to digest for 30 minutes at room temperature. Digestion was stopped with the addition of EDTA to 20mM and incubated for 5 minutes prior to dissociation of the remaining tissue through a wire sieve to generate a single cell suspension. Pelleted splenocytes were then treated with red blood cell lysis buffer (0.84% NH_4Cl) for 5 minutes and then washed. The remaining splenocytes were resuspended in wash buffer (HBSS, 2% FCS, 5mM EDTA) and layered over a 14.5% (w/v) Histodenz (Sigma, Oakville, ON) solution. The entire gradient was then overlaid with 1ml of FCS and

centrifuged for 20 minutes at 1100g. Cells at the FCS interface were collected, washed and counted prior to proceeding with preparation for flow cytometric analysis.

2.2.3 Dendritic cell maturation assays

In vivo activation of DCs was done by injection of mice with 25 μg of LPS (Sigma) intravenously through the tail vein. After 18 hours, the mice were sacrificed and the spleens harvested for collagenase digestion and Histodenz enrichment as described above. Co-stimulatory molecule expression on $CD11c^{hi}$ cells was analyzed by flow cytometry. For *in vitro* analysis of co-stimulatory molecule expression, day 7 cells were re-plated on tissue culture plates with or without 1 μg/ml of LPS (*E. coli* 055:B5, Sigma, Oakville, ON), or 100ng/ml of UltraPure LPS (*E.coli* 0111:B4, Invivogen, Burlington, ON) or 250ng/ml Pam_3Csk_4 (InvivoGen, Burlington, ON), or 1uM CpG-ODN 1668 (phosphorothioated 5'-TCCATGACGTTCCTGATGCT-3'), synthesized by UBC NAPS facility), or 20ug/ml polyI:C (InvivoGen, Burlington, ON), overnight and the non-adherent cells were analyzed by flow cytometry after 24 hours using CD11c as a marker for BMDC. For cytokine analysis, BMDC were purified from the day 8 cultures by positive selection for CD11c using MACS magnetic beads (Miltenyi Biotec) and the purity of $CD11c^{+}$ cells was always greater than 95%. Supernatants for cytokine analysis were obtained by incubating 2×10^5 BMDCs with titrated amounts of LPS (*E. coli* 055:B5, Sigma), Ultrapure LPS (*E. coli* 0111:B4), Pam_3Csk_4, poly I:C or CpG-ODN 1668 for 20-24 hours and supernatant stored at $-80^{o}C$ for subsequent ELISA assays.

2.2.4 Detection of cytokines

IL-12p70, IL-6 and TNFα cytokine secretion by BMDC was analyzed by ELISA (eBioscience, San Diego, CA) according to the manufacturer's instructions. The ELISA for IFNβ (106) used a mouse anti-IFNβ monoclonal (7F-D3, Abcam, Burlington, ON) for capture and a polyclonal rabbit anti-mouse IFNβ followed by goat anti-rabbit HRP for detection (PBL Biomedical Laboratories, Burlington, ON). For intracellular cytokine staining of splenic dendritic cells, collagenase digested single-cell spleen suspensions were plated at 1 x 10^7 cells/ml in 96-well tissue culture plates with or without 10 μg/ml of LPS (Sigma) for 5 hours with the addition of GolgiPlug (BD Bioscience) for the last 4 hours. FcRs were blocked and cells subsequently labeled with antibodies specific for CD11c, MHCII and DX5 prior to fixation and permeabilization (4% paraformaldehyde, 0.5% saponin and 2% FCS). The cell suspension was then incubated with cytokine-specific antibodies and the percentage of cytokine secreting cells was determined by flow cytometry where dendritic cells were identified as $CD11c^+MHCII^+DX5^-$.

2.2.5 T cell and NK cell activation assays

OTII CD4 T cells (10^5) were co-cultured with 5 x 10^4 $CD45^{+/+}$ or $CD45^{-/-}$ BMDC with the indicated amounts of $OVA_{323-339}$ peptide and 100ng/ml LPS (InvivoGen), or 250ng/ml Pam_3Csk_4 (Invivogen), or 1μM CpG in round-bottom 96-well plates for 3 days. Supernatants were harvested for IL2 and IFNγ ELISA (eBioscience). Co-cultures were then expanded into fresh media with 10 U/ml of recombinant mouse IL-2 (eBioscience) for

another 3 days. For IFNγ secretion, cells were washed and re-plated for activation with 20 ng/ml of PMA and 1 μg/ml ionomycin for 4 hours with the addition of GolgiPlug during the final 3 hours. Intracellular IFNγ in $CD4^+$ cells was analyzed by flow cytometry. NK cells were isolated from spleens of $CD45^{+/+}$ mice using MACS DX5 positive selection (Miltenyi Biotec.) and were >90% pure. Co-cultures were initiated with 2 x 10^5 purified NK cells and 1 x 10^5 $CD45^{+/+}$ or $CD45^{-/-}$ purified BMDC and incubated overnight in the presence of 1 μg/ml LPS (InvivoGen or Sigma). Supernatants were harvested and stored at -80°C prior to analysis of IFNγ by ELISA (eBioscience).

2.2.6 Immunoprecipitation and western blotting

For phosphotyrosine blotting of cell lysates, 1 x 10^6 cells were lysed in buffer containing 1% Triton-X 100, 20 mM Tris-HCL pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 0.2 mM sodium molybdate and 0.5 mM sodium orthovanadate (1%Tx-TNE). For immunoprecipitation of Lyn, Jak1 and Jak2, 1 x 10^7 stimulated cells were lysed in 1% NP-40 TNE buffer (20 mM Tris-HCL pH 7.5, 150 mM NaCl, 2 mM EDTA) containing 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 0.2 mM sodium molybdate and 0.5 mM sodium orthovanadate and immunoprecipitated with 1 μg of antibody. For immunoprecipitation of SHIP (Src homology domain containing 5'-inositol phosphatase), 1 x 10^7 cells were lysed in 0.5% NP-40, 100 mM sodium fluoride, 2 mM tetrasodium pyrophosphate, 2 mM sodium orthovanadate, 2 mM EDTA, 2 mM sodium molybdate and 50 mM HEPES (pH 7.3) and SHIP antisera was added. Immunoprecipitations

were run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidine fluoride (PVDF) membranes. For Western blotting, all primary antibodies were added at 1:1000 dilution in 0.1% BSA and incubated for 1 hour prior to the addition of HRP labelled secondary antibody for 30 minutes. Membranes were then washed at least 6 times in Tris-buffered saline solution (20 mM Tris pH 7.5, 150 mM sodium chloride) containing 1% Tween-20 and developed using enhanced chemiluminescence (GE Health Care, Piscataway, NJ).

2.2.6 NF-kB activation assays

Purified BMDC were activated with 100 ng/ml of Ultrapure LPS. Nuclear extracts were prepared by first lysing the outer membrane of stimulated cells with 10 mM HEPES (pH 7.9), 50 mM sodium chloride, 0.5 M sucrose, 0.1 mM EDTA, 0.5% Triton-X 100, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 µg/ml pepstatin. Nuclei were then pelleted at 2500g prior to lysis of the nuclear membrane in 10 mM HEPES (pH 7.9), 500 mM sodium chloride, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% NP-40, 0.2 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 µg/ml pepstatin. Equal amounts of nuclear extract were subjected to SDS-PAGE after protein concentration was determined by the bicinchoninic acid (BCA) assay. Proteins were transferred to PVDF membrane and NF-kB p65 was immunoblotted with a 1:1000 dilution of goat polyclonal anti-NF-kB p65 (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA) followed by rabbit anti-goat HRP. The NF-kB ELISA was performed as described [Renard, 2001 #7333]. Briefly, a biotinylated NF-kB consensus sequence (5'-

AGTTGAGGGGACTTTCCCAGGC) was immobilized on streptavidin plates and incubated with cell lysates. NF-kB p65 was detected using goat polyclonal anti-p65 (C-20) and rabbit anti-goat HRP from Jackson (Burlington, Ontario).

2.2.7 LPS models of septic shock

Age and sex-matched groups of adult mice were assembled for intraperitoneal injection. For the high-dose model, mice were injected with 10mg/kg of LPS (*E. coli* 055:B5, Sigma) and monitored for a period of 72 hours. Cumulative scores were established based on the severity of symptoms observed in the mice according to the UBC Rodent Monitoring Record for sick animals. Animals showing severe symptoms indicating an individual score of 6 according to criteria established on the UBC Rodent Monitoring Record were euthanized. For the low-dose sepsis model, age and sex-matched groups of mice were injected intraperitoneally with 20ug of UltraPure LPS (*E.coli* 0111:B4, Invivogen) and 20mg of the hepatotoxin d-galactosamine. Mice were monitored hourly and the endpoint was reached when the mice reached an individual score of 6 according to criteria established on the UBC Rodent Monitoring Record.

Serum TNFα was assessed in mice 1 hour or 2 hours post-injection with either 10mg/kg LPS or PBS. At the appropriate time points, animals were sacrificed and blood was collected by cardiac puncture and allowed to clot on ice. Clotted blood was centrifuged at 8,000rpm for 15 minutes in an Eppendorf benchtop centrifuge to separate the serum. Serum was harvested and stored at -80°C prior to analysis by cytokine ELISA.

2.2.8 Isolation of peritoneal cells

Peritoneal cells were collected by lavage of the peritoneal cavity with 5mls of a wash solution of HBSS/ 2% FCS/ 5mM EDTA. Aspirated cells were subjected to red blood cell lysis, washed and counted by hemocytometry. Enumerated peritoneal cells were prepared for analysis by flow cytometry as described above.

Chapter 3 CD45 in DC development and activation

3.1 Introduction

CD45 is expressed on the surface of all nucleated cells of the hematopoietic system, including DCs. The most extensive studies of CD45 function were performed in T and B cells where it was found to be necessary for proper antigen receptor signaling. However CD45 is expressed at high levels on myeloid cell types that do not possess antigen receptors. One aim of this study was to determine if CD45 was also critical for the development of dendritic cells. DCs can be derived from both myeloid and lymphoid progenitors. Although CD45 is required for the development of lymphoid cells, it is not required for the development of myeloid cells. It was therefore of interest to determine if CD45 would affect the development of DCs.

Little is known about the role of CD45 in DCs but SFK family members have been previously identified in DCs. SFK have also been implicated in the processes of adhesion, and LPS and CpG-mediated signaling cytokine secretion in macrophages. When DCs encounter PAMPs, signaling through the TLR activates the DCs and results in proinflammatory cytokine secretion and upregulation of costimulatory molecules on the surface of the DC. Since a role for SFK, especially Lyn, had previously been identified in TLR-induced cytokine secretion in both DC and macrophages, the second aim of this study was to determine what effect the absence of the master SFK-regulator, CD45, has in DC activation by PAMPs. Since Lyn can be a CD45 substrate, the hypothesis was that TLR-driven cytokine secretion would be decreased in the absence of CD45, similar to the Lyn knockout (107).

3.2 Results

3.2.1 *Splenic dendritic cell development is altered in the absence of CD45*

CD45 is capable of undergoing alternative splicing of exons 4-6 (also referred to as A, B and C), which can result in the expression of multiple isoforms including one isoform containing none of these exons (CD45RO). The expression of different isoforms of CD45 has been used to identify different populations of cells. In DCs, the presence of B220 (a glycosylated form of CD45RABC) marks the plasmacytoid population while the expression of high levels of the CD45RB isoform has been associated with a population of tolerogenic DCs. It was initially of interest to fully characterize CD45 expression on splenic DCs in wild type, C57BL/6 mice.

Splenic DCs were partially purified from collagenase D digested spleens of adult (over 6 weeks old) C57BL/6 mice by density-gradient centrifugation in Histodenz. The cell fraction containing the DCs was harvested from the top of the gradient and immediately processed for flow cytometric analysis. Gates were drawn to select $CD11c^+$ positive cells and expression of CD45RA, CD45RB (23G2), CD45RC or B220 was assessed. Splenic dendritic cells were found to have populations that were positive for the A, B and B220 isoforms tested (fig 3.1). The negative control peak overlapped almost entirely with CD45RC peak. It was not possible to determine CD45RO expression since it is an absence of any of the other isoforms. These results disagree somewhat with a paper published by Haidl *et al*., (108) where only CD45RB and CD45RO were detected. One key difference is likely to be the isolation methods. The original study enriched for DCs on a BSA gradient followed by

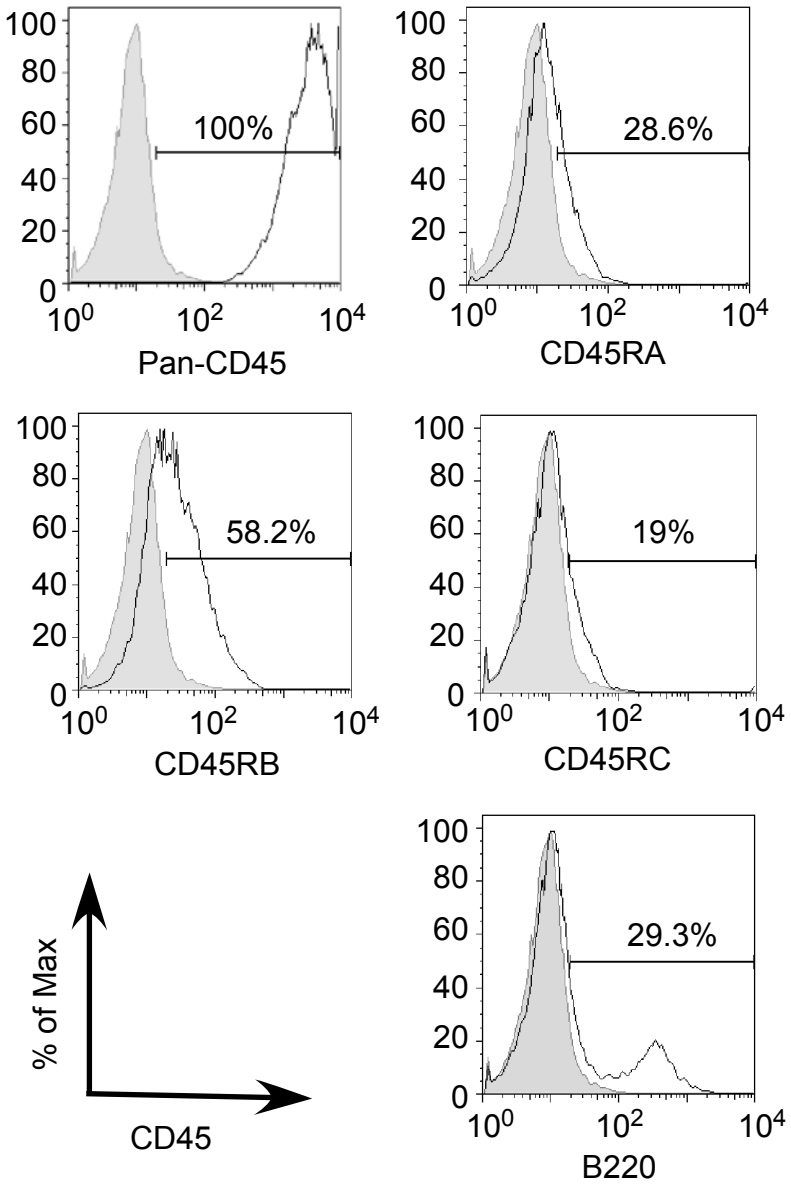
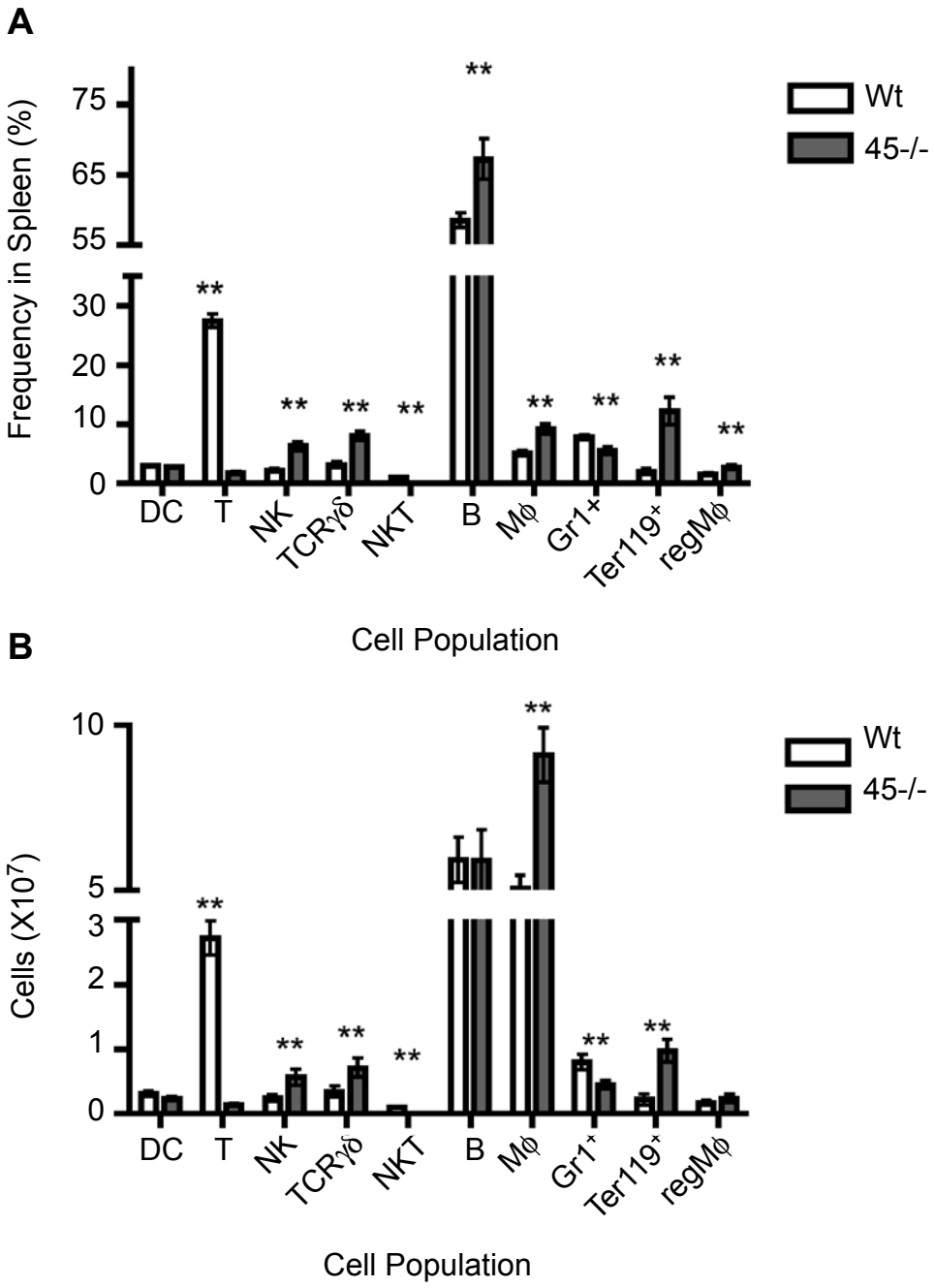


Fig. 3.1 CD45 isoform expression on splenic dendritic cells. Splenic dendritic cells were partially purified on a Histodenz density gradient and analyzed by flow cytometry for CD45 expression on $CD11c^{+}$ gated cells. A fluorescence-minus-one negative control (filled histogram) was used to define the CD45 labeled, percentages positive (open histogram). Experiment is a representative of 3 independent replicates.

plastic adherence whereas the work presented here did not culture the cells prior to flow cytometric analysis. It is possible that during the BSA gradient or the plastic adherence step, the pDC population that expresses B220 (and thus the RA and RC isoforms) was lost.

Having established that CD45 is expressed on splenic DCs in wild-type mice, it was then of interest to determine if the absence of CD45 would hinder or alter the development of splenic DC. Splenocyte suspensions were obtained after collagenase digestion from both C57BL/6 ($CD45^{+/+}$) and CD45 exon-9 knockout mice backcrossed to 9 generations (hereafter referred to as $CD45^{-/-}$) and analyzed by flow cytometry for cells expressing high levels of the dendritic cell marker, CD11c, as well as for MHCII (fig 3.2A). $CD45^{-/-}$ mice had a slightly reduced frequency of dendritic cells (identified as $CD11c^{hi}$ $MHCII^{hi}$), but this was not significant when averaged over several experiments (fig 3.2B). There was a slight increase in the absolute number of splenic dendritic cells isolated from $CD45^{-/-}$ mice compared to $CD45^{+/+}$ ($3.8 \pm 2.3 \times 10^6$ vs. $2.5 \pm 1.6 \times 10^6$, n=5) (fig 3.2D), which was likely due to the two-fold increase in the total cellularity of the $CD45^{-/-}$ spleen ((89, 90) and fig 3.2C.).

Although CD45 has a dramatic effect on the development of T and B cells, it has not been shown to grossly alter the development of other immune cell populations. In $CD45^{-/-}$ mice, macrophages are present ((97) and fig 3.3), natural killer cells are present (although the population is expanded about 2-fold ((109) and fig 3.3)), granulocytes (fig 3.3) and dendritic cells are all present (fig 3.3). CD45 is present on early stem-cell progenitors but its absence



3.3 Characterization of the splenic composition in CD45$^{-/-}$ mice compared to C57BL/6. Single cell suspensions were generated from collagenase D digested spleens from CD45$^{+/+}$ (Wt) and CD45$^{-/-}$ mice. Splenocytes were enumerated by hemocytometry and labeled for flow cytometric analysis of the different cell populations. (A) Graphical representation of the frequencies of the individual populations present in the spleens and (B) shows the absolute numbers of the cells in each population based on the total number of cells recovered. Graphs are the mean $\pm$SEM from 6 mice collected in 3 independent experiments. In both panels, ** denotes $p \leq 0.01$.

has not been shown to have a dramatic effect on the development of these other cell types that not dependent on antigen-receptor signaling.

As previously mentioned, different functional populations of DCs can be identified based on their expression of different CD45 isoforms. It was possible that the complete absence of CD45 could differentially impact the DC subset distribution. Splenic DCs can be very broadly divided into two subsets based on the mutually exclusive expression of CD8α or CD11b. Several studies have suggested that these subsets are functionally distinct (1, 2, 26). Splenocyte suspensions were subjected to Histodenz density gradient centrifugation and analyzed by flow cytometry for expression of CD11b and CD8α on $CD11c^{hi}$ cells. The distribution of these two subsets was skewed towards the $CD8\alpha^{+}$ population in the absence of CD45 (fig 3.4).

A third but distinct population of DCs is also present in the spleen, the plasmacytoid DCs (pDCs). The classical marker for pDCs is the CD45 B220 isoform. Since $CD45^{-/-}$ mice do not express B220, they were identified using the mPDCA-1 antibody (Miltenyi Biotec) and were $CD11c^{lo}$. The lack of B220 expression on $CD45^{-/-}$ dendritic cells did not affect the percentage of plasmacytoid dendritic cells present in the spleen of $CD45^{-/-}$ mice (fig 3.5). The lack of CD45 results in alterations in splenic cellularity, composition and architecture (110). It was plausible that this could result in alterations of the chemokine and cytokine milieu in the spleen and thus affect the development of the splenic DC populations to favor the CD8α subset over the CD11b subset. To determine if the populations skewing was a result of T cell paucity and an increase in immature B cells, we crossed the $CD45^{-/-}$ mice onto the RAG2 knockout background. This enabled the comparison of $CD45^{+/+}$ spleens

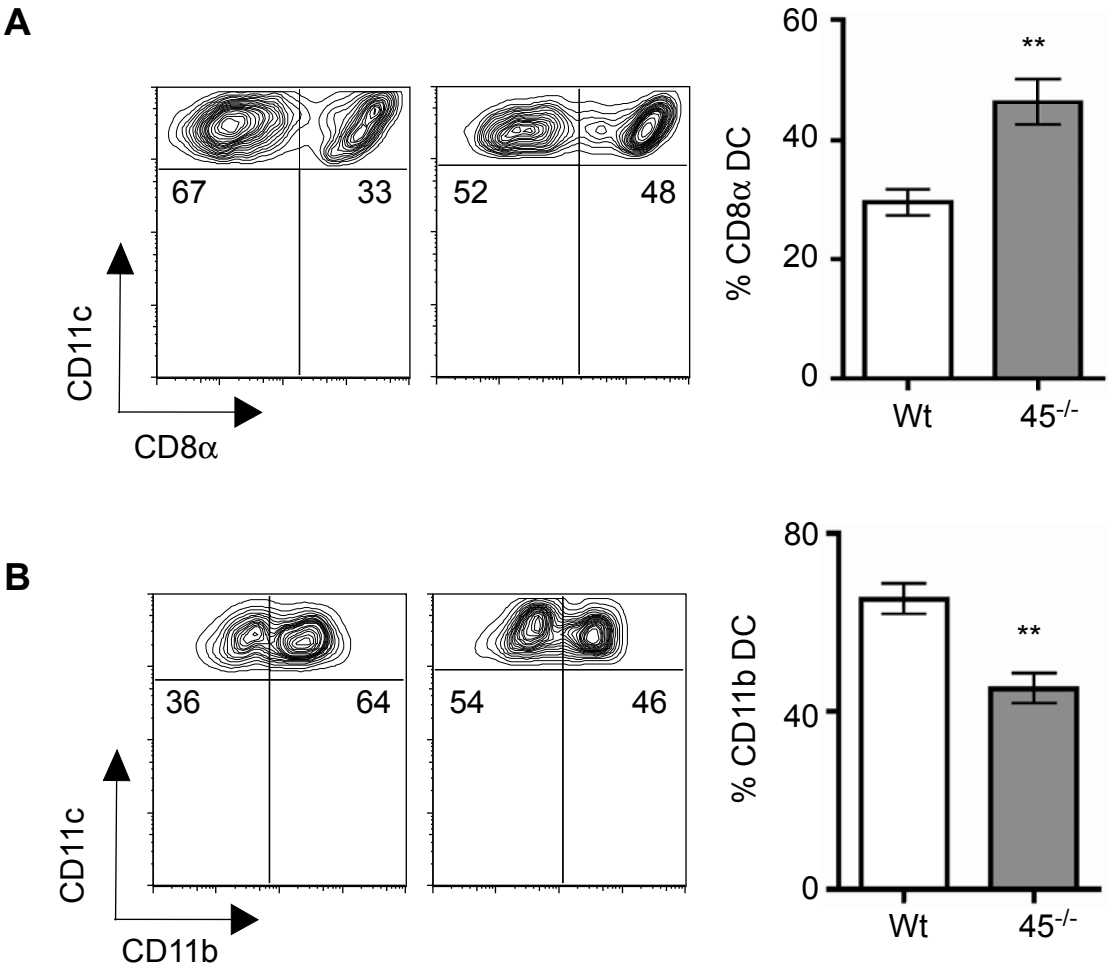


Fig 3.4 Splenic dendritic populations in the absence of CD45. Partially purified splenic dendritic cells from wild type and $CD45^{-/-}$ mice were analyzed by flow cytometry. Expression of (A) CD8α and (B) CD11b on $CD11c^{+}$ cells was determined. Graphs on the right indicate that the CD8α+ population was significantly increased in $CD45^{-/-}$ mice ($p=0.003$, $n=5$) while the CD11b+ population was significantly decreased ($p=0.001$, $n=6$). FACS plots are one representative of 3 experiments and the graphs are the mean ± SEM.

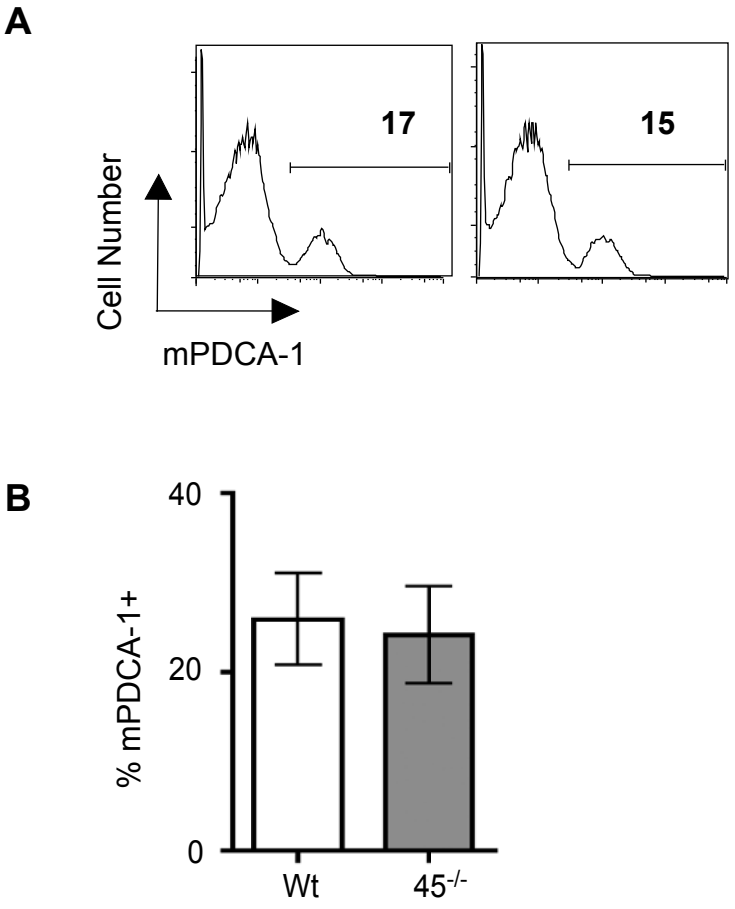


Fig 3.5 Plasmacytoid DC population frequency in the absence of CD45. (A) Splenic dendritic cells were enriched from wild type and $CD45^{-/-}$ mice and analyzed by flow cytometry for expression of mPDCA-1. A representative histogram plot from one experiment is shown. (B) Experimental summary of three independent experiments (mean $\pm$ SEM) showing that there is no significant difference in the frequency of pDC in $CD45^{-/-}$ spleens.

lacking T and B cells with $CD45^{-/-}$ spleens lacking T and B cells, in effect controlling for the differences in these populations seen when C57BL/6 mice were compared with $CD45^{-/-}$ mice. On a $RAG2^{-/-}$ background, $CD45^{-/-}$ mice showed an increase in the frequency and numbers of the $CD8\alpha^{+}$ subset and a substantial decrease in the frequency and numbers of the $CD11b^{+}$ subset (more specifically the $CD4^{+}$ $CD11b^{+}$ subset) (fig 3.6). Interestingly, there was also an increase in the number of pDC in the $CD45^{-/-}RAG^{-/-}$ spleens, which was not consistently observed in the normal $CD45^{-/-}$ mice. This suggests that CD45 may have an effect on pDC homeostasis.

3.2.2 Bone marrow-derived dendritic cell development is not altered in the absence of CD45

To compare the *in vitro* development of $CD45^{+/+}$ and $CD45^{-/-}$ DCs, bone marrow was isolated from the respective mice and cultured in GM-CSF containing media, essentially as described (104), and the production and maturation of bone marrow-derived DCs (BMDC) was monitored over time. Figure 3.7A shows a decrease in the percentage of non-adherent bone marrow cells expressing the granulocyte marker, Ly6G, together with the concomitant increase in cells expressing CD11c for both $CD45^{+/+}$ and $CD45^{-/-}$ mice. By day 8 there were approximately equal percentages of non-adherent, $CD11c^{hi}$ cells from the bone marrow of wild-type and $CD45^{-/-}$ mice (fig. 3.7B), but the yield was slightly higher from $CD45^{-/-}$ bone marrow (fig. 3.7C). As the $CD11c^{hi}$ cells mature *in vitro*, they also become increasingly $MHCII^{hi}$ (fig. 3.7D) and by day 8 there was also a slight increase in the percentage of $CD45^{-/-}$ $MHCII^{hi}$ cells. Thus in terms of numbers and percentages, the lack of CD45 did not grossly

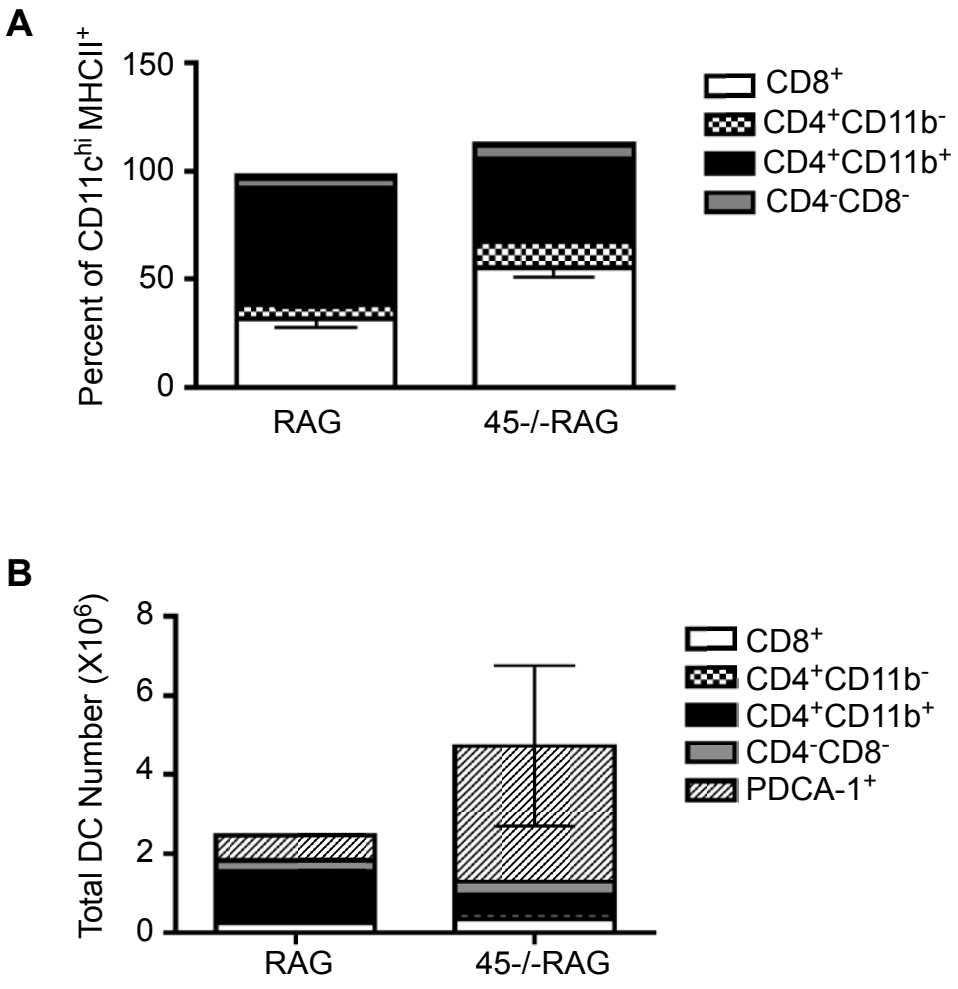


Fig 3.6 Development of CD45 $^{+/+}$ andCD45$^{-/-}$ splenic dendritic cell populations in the absence of T and B cells. CD45$^{-/-}$ mice were crossed onto the RAG2$^{-/-}$ background and splenic DC populations in these mice were compared to those in CD45$^{+/+}$RAG2$^{-/-}$ mice. (A) Graphical representation of splenic dendritic cell (CD11c^{hi} MHCII^{+}) frequencies obtained by flow cytometric analysis of splenocytes obtained from CD45$^{+/+}$RAG2$^{-/-}$ and CD45$^{-/-}$RAG2$^{-/-}$ mice. The graph is the mean $\pm$SEM from 3 individual experiments using 2 mice each. (B) Total numbers of the dendritic cell populations obtained by multiplication of the population percentage by the total number of splenocytes obtained after collagenase D digestion. The graph is the mean $\pm$SEM from 2 individual experiments using 2 mice each.

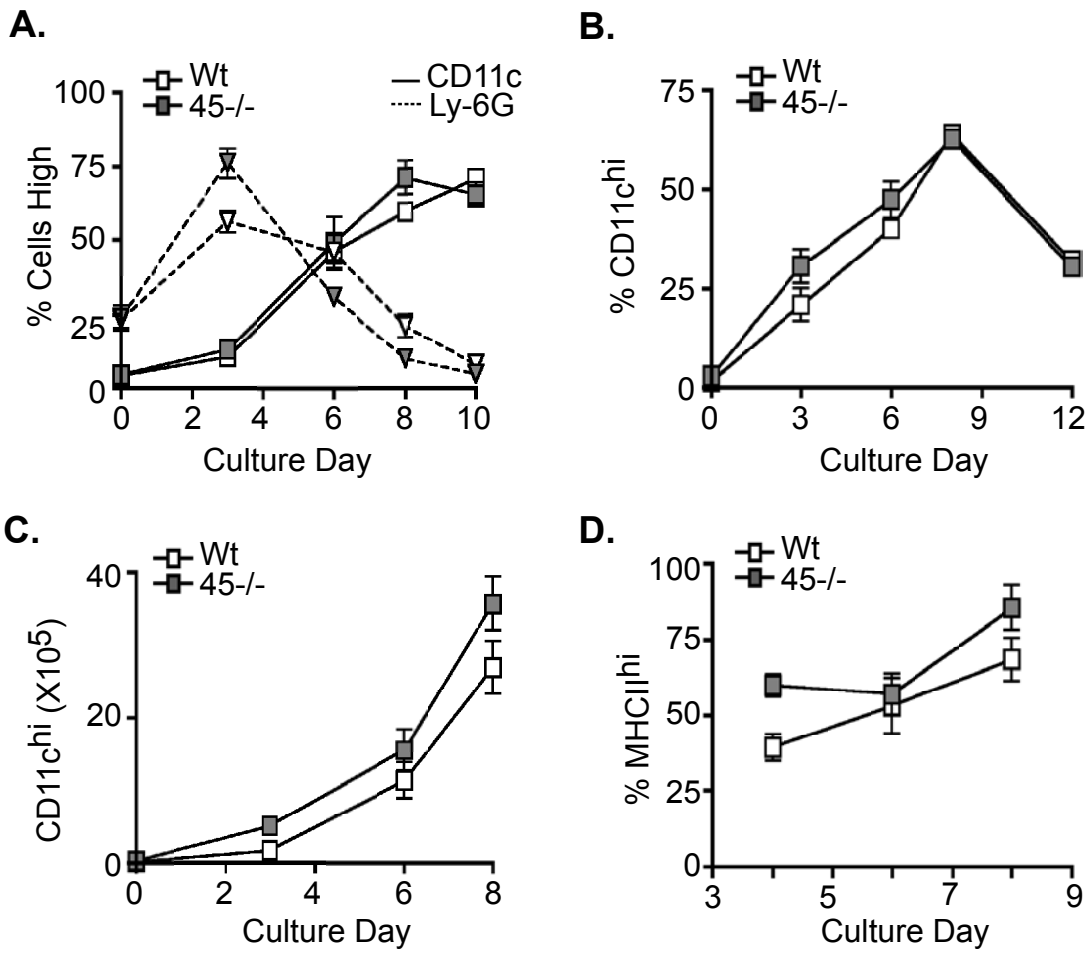


Fig 3.7 CD45$^{-/-}$ bone marrow can be differentiated into dendritic cells in GM-CSF culture. Graphical representation of BMDC development from CD45$^{+/+}$ (Wt) and CD45$^{-/-}$ mice. Bone marrow was cultured in media with GM-CSF for the indicated times and the non-adherent cells were harvested for analysis. (A) Analysis of granulocyte contamination (Ly-6G) as compared to BMDC generation (CD11c) in the non-adherent population over time. The percentage of cells highly expressing each marker is represented at each time point. (B) The percentage of CD11c^{hi} cells over time was obtained by flow cytometric gating of CD11c^{hi} cells present in the culture and (C) total numbers of CD11c^{hi} cells were obtained by multiplying the percentage of CD11c^{hi} cells by the total number of non-adherent cells. (D) Percentage of CD11c^{hi} cells that are also MHCII^{hi} over time. Experiments are the mean $\pm$ SEM from three independent experiments using 1 mouse each.

affect the *in vitro* generation of DCs, but did subtly increase the number of $CD11c^{hi}$ $MHCII^{hi}$ cells. There were no obvious differences in the morphology of these dendritic cells (data not shown).

CD45 has been shown to regulate integrin signaling in macrophages (111). Cultured bone marrow-derived macrophages lacking CD45 show a very tight initial adherence but then over time they become less adhesive. Experiments with peritoneal macrophages showed that $CD45^{-/-}$ cells did not adhere in overnight culture and showed a more rounded phenotype instead of the elongated and spread phenotype seen in $CD45^{+/+}$ cultures (data not shown). Although the BMDC used in these experiments were harvested from the non-adherent fraction, it was also of interest to determine if an effect of CD45 on BMDC development could be seen in the fraction of adherent cells in GM-CSF culture. At early time points in the culture (days 0-6) there was an increase in frequency (fig 3.8A) and numbers (fig 3.8B) of $CD11c^{hi}$ cells in the adherent fraction. By day 8 and onwards the difference was negated, suggesting that CD45 may be a negative regulator of adhesion in earlier BMDC progenitors but as more mature BMDC develop, they accumulate at the same percentages and numbers in the non-adherent fraction in both $CD45^{+/+}$ and $CD45^{-/-}$ cultures.

Other studies have identified a population of tolerogenic BMDC identified by their expression of high levels of the CD45RB isoform (28). To determine if the absence of CD45 would affect the ability of BMDC to activate or tolerize T cells, we first sought to identify which isoforms of CD45 are present on BMDC in C57BL/6 mice. In contrast to the study by Wakkach *et al*., (28) BMDC were not found to express high levels of any of the alternatively spliced exons of CD45 although they do express high levels of CD45 (fig 3.9). Although a uniformly low level of CD45RB was detected, no distinct population expressing a very high

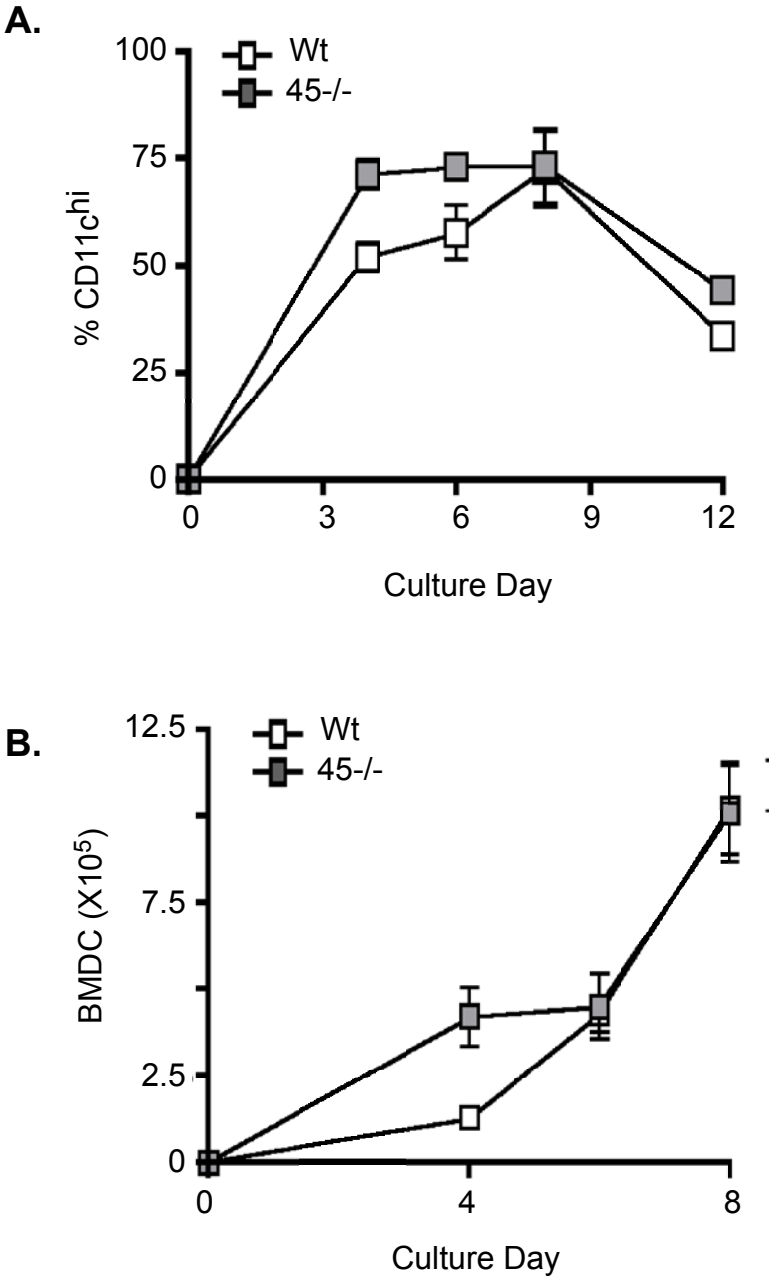


Fig 3.8 Effect of the lack of CD45 on the adherent fraction of the GM-CSF culture at day 8. Graphical representation of BMDC development from $CD45^{+/+}$ (Wt) and $CD45^{-/-}$ mice. Bone marrow was cultured in media with GM-CSF for the indicated times and the adherent cells were harvested for analysis at the indicated timepoints. (A) BMDCs are present in the non-adherent fraction of $CD45^{-/-}$ cultures at the same frequency as the wt by day 8 and in (B) similar numbers. Experiments are the mean $\pm$ SEM from three independent experiments using 1 mouse each.

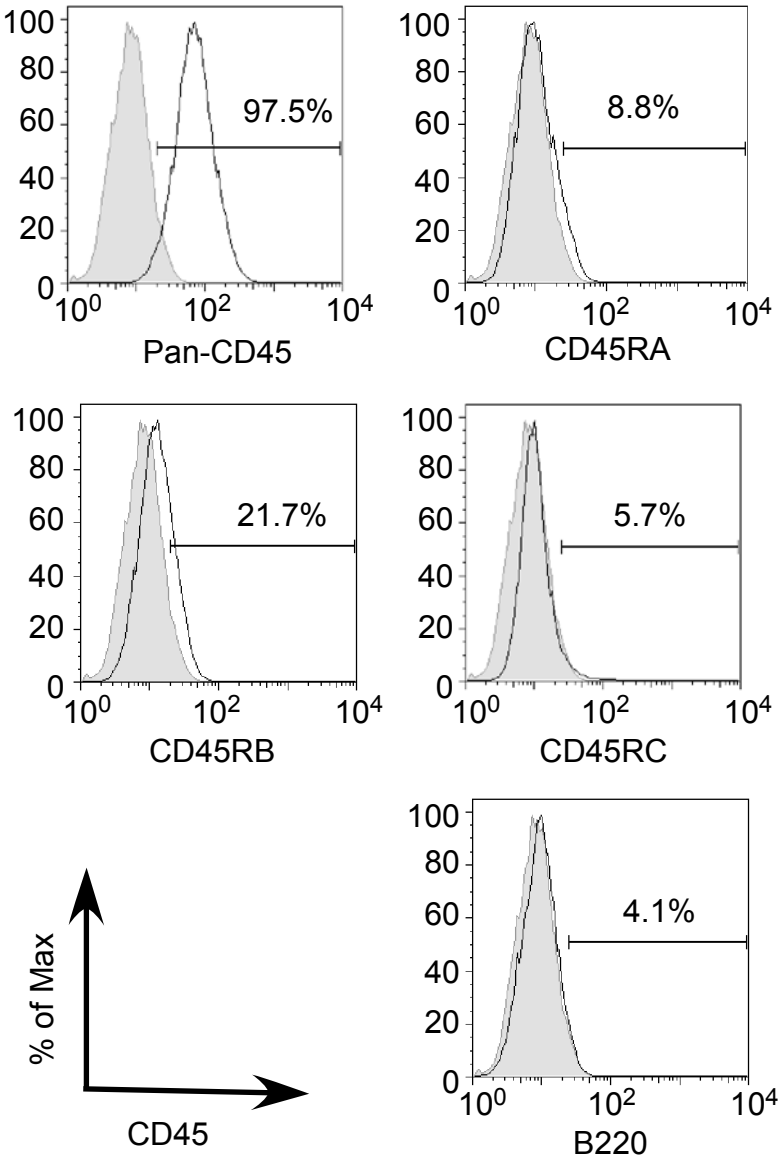


Fig 3.9 CD45 isoform expression on GM-CSF cultured BMDCs from C57BL/6 mice. BMDC cultures were harvested at day 8 and analyzed by flow cytometry for expression of CD45 and its isoforms on gated $CD11c^{+}$ cells. The percentage of positive cells is displayed to the right of the histogram. The grey histogram is the staining for the isotype control Experiment is one representative of 2 independent experiments.

level of CD45RB was observed. This discrepancy could possibly be a result of the genetic background of the mice (C57BL/6 vs. BALB/c) or differences in culture conditions (Wakkach's group added both TNFα and GM-CSF to the BMDC culture). Even with the addition of IL-10 to the BMDC culture, stated to expand the population of $CD45RB^{hi}$ BMDC from 1% to 15%, no $CD45RB^{hi}$ BMDC were detected (data not shown).

3.2.3 CD45 affects co-stimulatory molecule expression in splenic and BMDC.

DCs express surface costimulatory molecules, such as the B-7 family members CD80 and CD86, to aid in the activation of naïve T cells. Immature DCs that have not encountered pathogen-derived products express low levels of these molecules to prevent inappropriate T cell activation while activated and mature DCs express these molecules at high levels in order to prime an immune response. Although DC do develop in the absence of CD45, it was necessary to determine if they were phenotypically normal in this aspect.

Analysis of Histodenz enriched splenic $CD45^{-/-}CD11c^{hi}$ DCs showed a slight increase in expression of co-stimulatory molecules. CD80 showed a two-fold increase in expression, while levels of CD40 and CD86 were only slightly elevated in the $CD45^{-/-}$ DCs. A slight decrease in MHCII expression was observed (fig. 3.10, upper panels and graph).

Flow cytometric analysis of the expression levels of the co-stimulatory molecules (CD40, CD80 and CD86) and MHCII on unstimulated day 8 BMDC (non-adherent, $CD11c^{hi}$) revealed a higher level of expression of all these molecules on the $CD45^{-/-}$ DCs (fig 3.11, upper panels and graph), although the levels of MHCII were more variable.

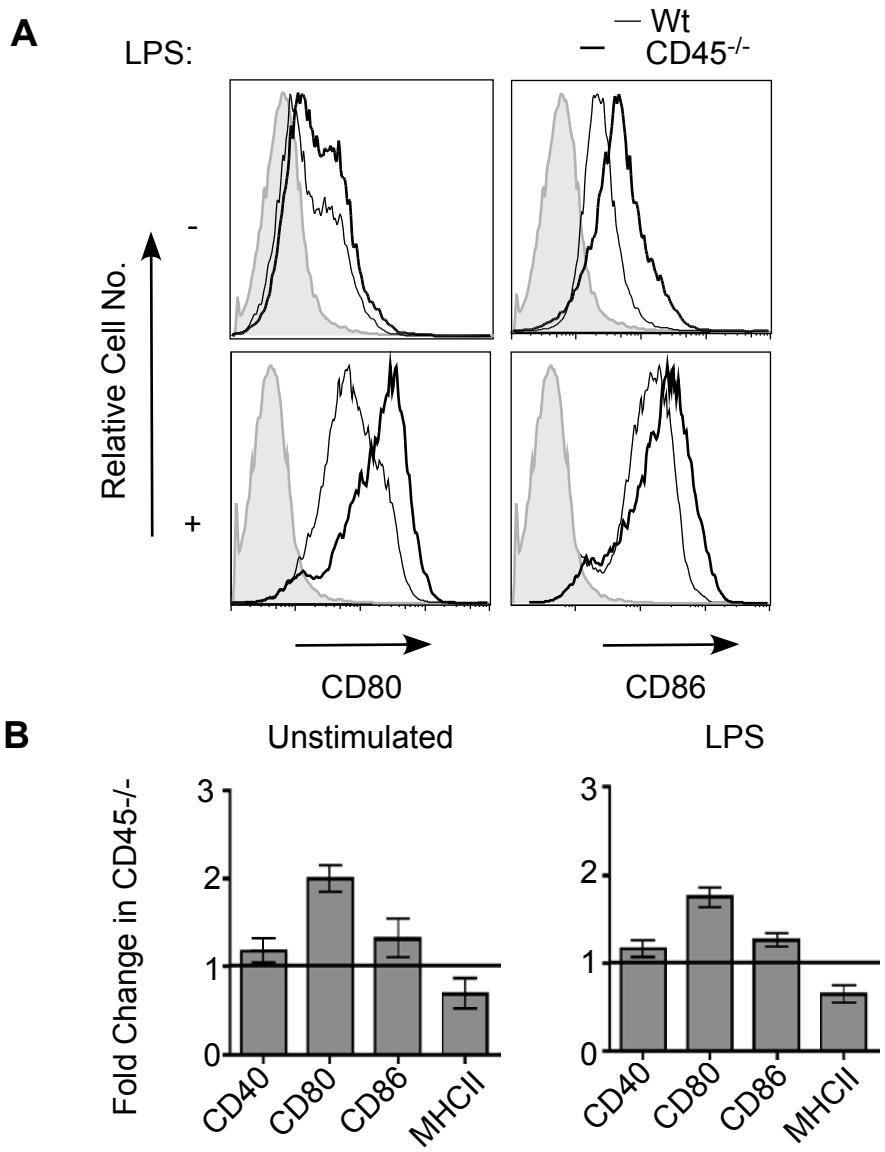


Fig 3.10 Costimulatory molecule expression and upregulation in CD45$^{+/+}$ and CD45$^{-/-}$ splenic DCs in response to LPS. (A) Co-stimulatory molecule expression on unstimulated and *in vivo* activated splenic dendritic cells. CD45$^{+/+}$ (thin line) and CD45$^{-/-}$ (thick line) mice were injected either with PBS (top panel) or 25 μg of Sigma LPS (bottom panel). Spleens were harvested after 18 hours and DCs were enriched by separation on Histodenz. The expression of CD80 and CD86 on CD11c^{hi} cells was analyzed by flow cytometry. Expression levels are shown on a log scale and the grey histogram is the negative control. One representative experiment of three is shown. Each experiment used 2 mice per condition. (B) The graph depicts the mean fold difference (± SEM) in co-stimulatory molecule expression on CD45$^{-/-}$ compared to CD45$^{+/+}$ DCs over three experiments.

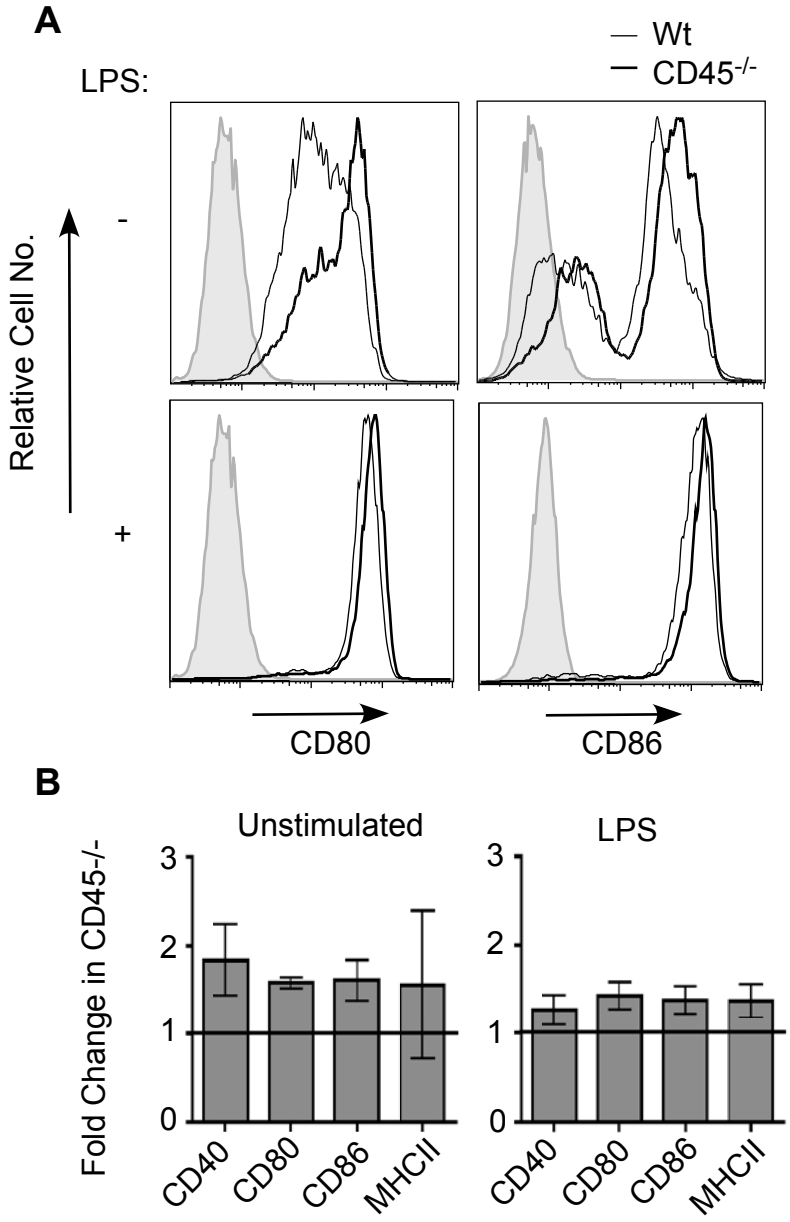


Fig 3.11 CD45$^{-/-}$ BMDCs express costimulatory molecules and upregulate them in response to LPS. (A) CD45$^{+/+}$ (Wt, thin line) and CD45$^{-/-}$ (thick line) bone marrow cells from day 7 GM-CSF cultures were incubated overnight with or without (bottom and top panels, respectively) 1 μg/ml Sigma LPS prior to analysis of CD80 and CD86 on CD11c^{hi} cells by flow cytometry. Expression levels are shown on a log scale. Flow cytometry experiments are representative of three independent experiments using 1 mouse each. (B) Graphs show the average fold difference in mean fluorescence intensity ± SEM for CD40, CD80, CD86 and MHCII from three experiments.

LPS is a potent pathogen-derived stimulator of DCs and both *in vitro* exposure of BMDC and *in vivo* exposure of splenic DCs to LPS resulted in a substantial upregulation of CD40, CD80, CD86 and MHCII on both the $CD45^{+/+}$ and $CD45^{-/-}$ dendritic cells. Despite this, expression levels of the co-stimulatory molecules still remained slightly higher in the $CD45^{-/-}$ splenic and BMDC (figs. 3.10 and 11, lower panels). This implies that CD45 may have a minor role in maintaining the levels of costimulatory molecule expression.

3.2.4 CD45 is a positive regulator of TLR4-driven proinflammatory cytokine production

In addition to increasing the expression of co-stimulatory molecules on the DC surface, pathogen-derived products, like LPS, signal through Toll-like receptors (TLRs) to induce the expression of proinflammatory cytokines such as IL-12, IL-6 and TNFα. These cytokines play an important role in shaping the adaptive immune response. CD45 substrates, the Src-family of protein tyrosine kinases (SFKs), have been shown to influence TLR-mediated cytokine secretion.

The activity of Src-family kinases are required for CpG induced TNFα release and inducible nitric-oxide synthase accumulation in RAW cells (112). PP1 and PP2, specific inhibitors of SFKs, can block LPS-mediated IL-12 secretion in human monocyte-derived DCs (113), while the phosphatase inhibitor, sodium orthovanadate can enhance LPS-induced TNFα secretion. A couple of groups have shown that both Lyn and Hck phosphorylation occur after LPS stimulation (114) (115). In human peripheral monocytes, anti-CD45 antibodies enhance the respiratory burst induced by LPS (116) and caused differentiated, LPS stimulated, THP-1 cells to increase their TNFα secretion (117).

In splenic DCs, the $CD8\alpha^+$ subset is responsible for secretion of high levels of proinflammatory cytokines following TLR engagement. In the $CD45^{-/-}$ mice, this subset is preferentially expanded so it was of interest to determine if cytokine secretion following LPS stimulation (through TLR4) would be affected. $CD45^{+/+}$ and $CD45^{-/-}$ splenocytes were stimulated *in vitro* with LPS for 5 hours and intracellular cytokine production was examined in the dendritic cells ($CD11c^{hi}MHCII^{hi}DX5^-$) by flow cytometry (fig. 3.12A). The percentage of cells positive for IL12p40 and TNFα was noticeably lower in the $CD45^{-/-}$ DCs and this was significant ($p = 0.03$ and 0.01 for TNFα and IL-12 respectively) when averaged over at least 3 independent experiments (fig 3.12B).

A similar reduction in IL-12p70, TNFα and IL-6 cytokine production was also observed when 1 μg/ml of LPS was used to stimulate BMDC. The concentration of cytokine in the supernatant was measured by ELISA after 16-24 hours of stimulation and figure. 3.13A shows the average cytokine secreted from three independent experiments with 1 mouse each. To further determine a specific effect on TLR4 signaling, a titration was performed on BMDC with Ultrapure LPS (Invivogen).

This revealed a more subtle difference between $CD45^{+/+}$ and $CD45^{-/-}$ DCs that was dependent on the LPS concentration (fig. 3.13B). At low levels (10 ng/ml) of LPS, $CD45^{-/-}$ DCs showed a slight increase in IL-12p70 secretion and a similar trend but no reproducible difference in TNFα or IL6 secretion. However, at higher LPS concentrations the trend was reversed. A slight, but significant, reduction in all three proinflammatory cytokines was reproducibly seen at 100 ng/ml LPS but this was not consistently seen at 1 μg/ml of LPS. The data shown in fig 3.13B is a representative experiment showing the average cytokine production from three mice per condition. This difference could not be attributed to

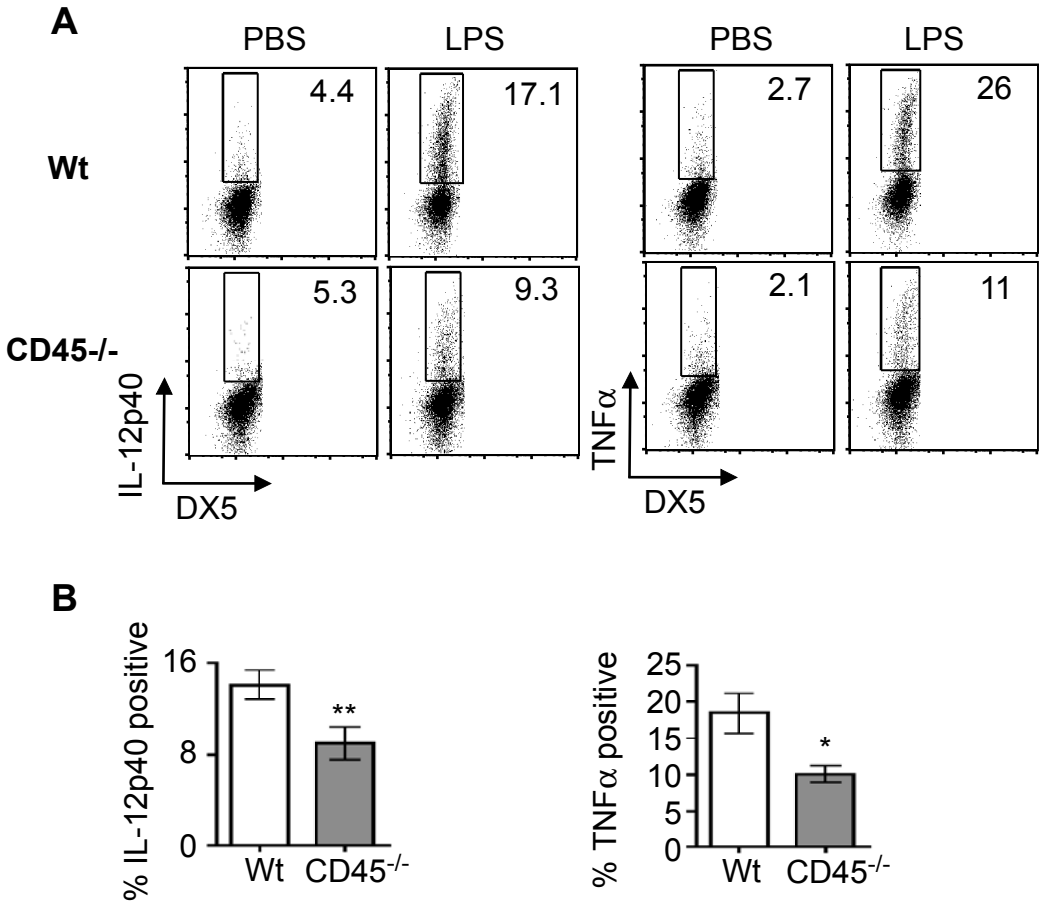


Fig 3.12 Proinflammatory cytokine secretion in response to LPS in CD45$^{-/-}$ splenic dendritic cells. (A) Flow cytometric analysis after *in vitro* stimulation of splenocytes with 10 μg/ml LPS for 5 hours. IL12p40 and TNFα production were assessed by flow cytometry of CD11c^{hi} MHCII^{hi} DX5^{-} cells. Experiment is representative of 3 or 4 independent replicates and (B) the mean ± SEM from all the experiments is shown graphically below. The percentages of IL-12p40 and TNFα producing cells were significantly reduced (**p=0.01, *p=0.03 respectively) in the CD45$^{-/-}$ spleens.

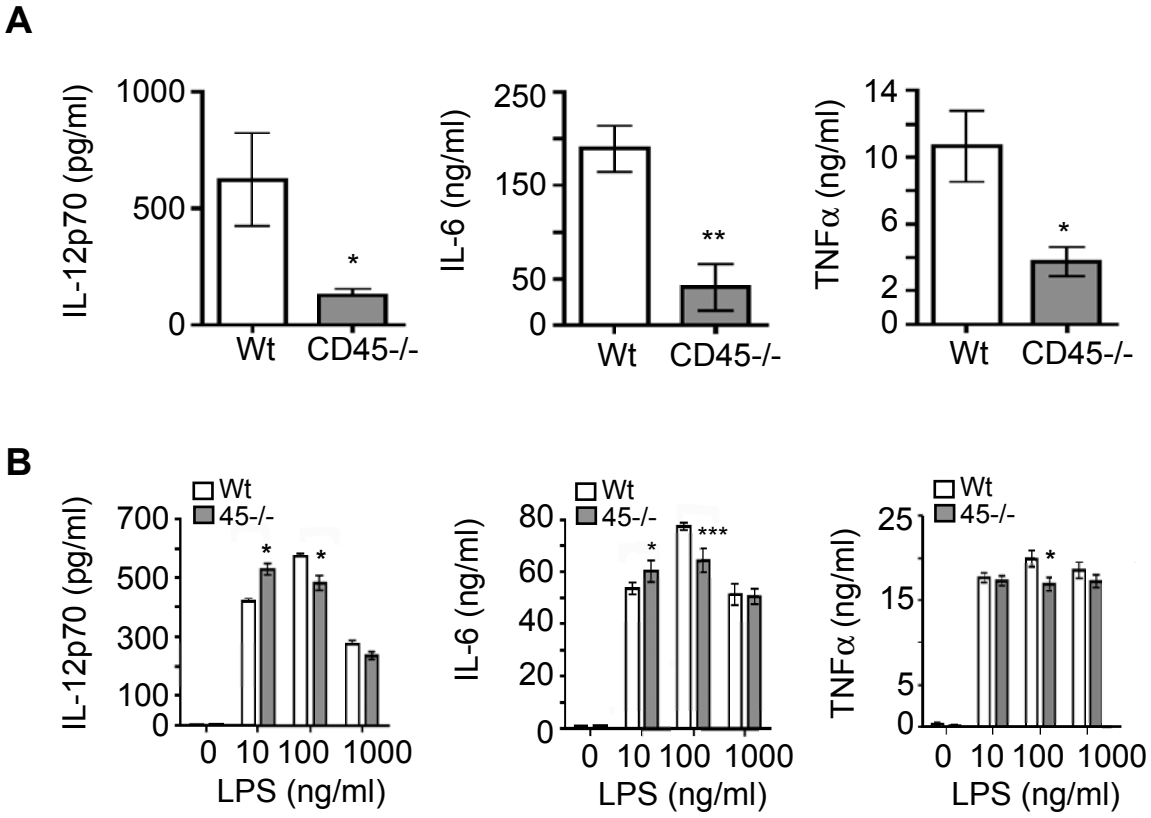


Fig 3.13 Proinflammatory cytokine secretion in response to LPS from CD45$^{-/-}$ BMDC cultures (A) *In vitro* stimulation of BMDC with 1 μg/ml Sigma LPS. After 22 to 24 hours of stimulation, the amounts of IL-12p70, TNFα and IL-6 in the culture supernatant were determined by ELISA. Data are the mean ± SEM of 3-5 independent experiments with 1 mouse each. (B) *In vitro* stimulation of BMDC with titrated amounts of Ultrapure LPS. After 22 to 24 hours of stimulation, the amounts of IL-12p70, TNFα and IL-6 in the culture supernatant were determined by ELISA. One representative experiment of the average from 3 mice ± SEM is shown and was repeated three times, * $p < 0.05$, ** $p \leq 0.01$, *** $p < 0.001$.

differences in TLR4 expression, as $CD45^{+/+}$ and $CD45^{-/-}$ DCs expressed similar levels when assessed by FACS analysis (fig 3.14) This indicates that the absence of CD45 in dendritic cells can affect the amount of proinflammatory cytokine produced by both splenic and BMDC in response to bacterial LPS.

3.2.5 CD45 is a negative regulator of TLR2 and TLR9-mediated proinflammatory cytokine secretion

LPS signaling through TLR4 is an extensively used model system to look at the activation of immune cells like DCs. However DCs express many other TLRs capable of recognizing things other than Gram-negative bacterial cell walls. For example, TLR2 recognizes components of Gram-positive bacterial cell walls; TLR3 recognizes double-stranded RNA and TLR9 recognizes unmethylated CpG DNA. Although the structure of the TLRs is similar, TLR4 and TLRs 2 and 9 have differences in their signaling pathways. TLR4 signaling uses both a MyD88-dependent and a MyD88-independent pathway, whereas TLR2 and TLR9 are both strictly dependent on MyD88 and TLR3 uses only a MyD88-independent pathway.

To determine if $CD45^{-/-}$ DCs can respond to other TLR ligands, BMDC were first incubated for 20-22 hours in the presence of Pam_3Csk_4 (a synthetic TLR2 ligand), CpG ODN 1668 (TLR9) or the synthetic TLR3 ligand, polyinosinic:polycytidylic acid (poly I:C) to assess their ability to upregulate their costimulatory molecule. $CD45^{-/-}$ BMDC were able to upregulate the costimulatory molecules CD80 and CD86 to levels similar to those observed in $CD45^{+/+}$ BMDC (fig. 3.15).

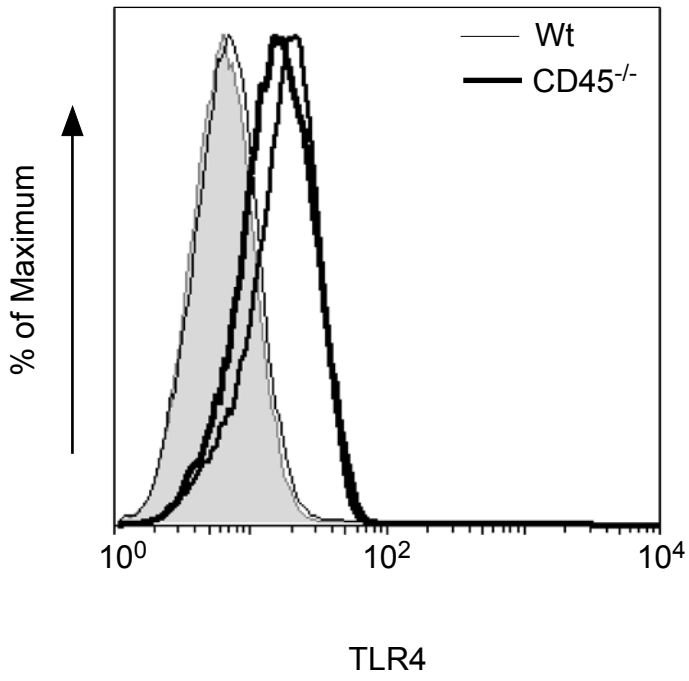


Fig 3.14 TLR4 expression on $CD45^{-/-}$ and $CD45^{+/+}$ BMDC. BMDCs from day 8 BMDC cultures were analyzed by flow cytometry to determine the surface levels of TLR4 on the $CD11c^{+}$ fraction. The shaded histograms are the isotype controls for wild type and $CD45^{-/-}$ samples, the unfilled histogram with the light line represents C57BL/6 BMDC labeled with anti-TLR and the unfilled histogram with the bold line is comparable label on the $CD45^{-/-}$ BMDCs.

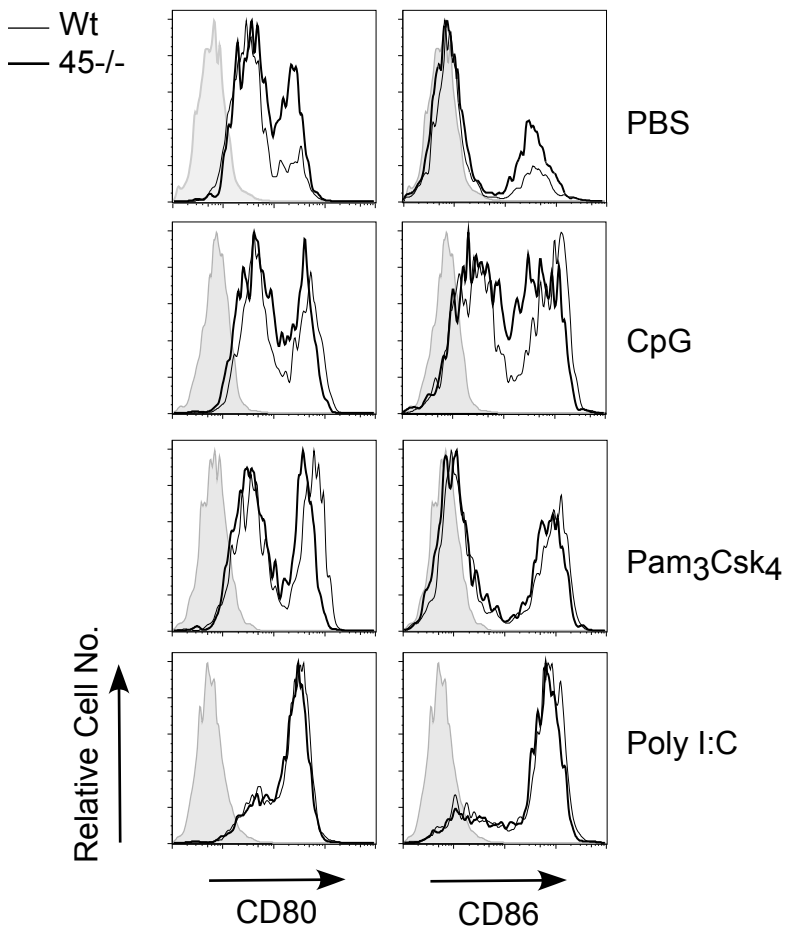


Fig 3.15 Upregulation of costimulatory molecules on CD45$^{-/-}$ BMDC TLR2, TLR3 and TLR9 ligands. CD45$^{+/+}$ (Wt, thin line) and CD45$^{-/-}$ (thick line) bone marrow cells from day 7 GM-CSF cultures were incubated overnight with or without (bottom and top panels, respectively) 1μM CpG, 250ng/ml Pam_3Csk_4, or 20ug/ml poly I:C prior to analysis of CD80 and CD86 on non-adherent CD11c^{hi} cells by flow-cytometry. Expression levels are shown on a log scale. Flow cytometry experiments are representative of three independent experiments using BMDC generated from a pool of 3 mice.

In contrast to LPS, $CD45^{-/-}$ BMDC produced more proinflammatory cytokines (IL-12, TNFα and IL-6) in response to the synthetic TLR2 ligand, Pam_3Csk_4 (fig. 3.16A) and in response to the TLR9 ligand, CpG 1668 (fig. 3.16B). No proinflammatory cytokine was secretion was detected for BMDC incubated with poly I:C (data not shown). The data are representative of three experiments and show the average cytokine produced from the BMDC from three individual mice. This increase in IL-12p70, IL-6 and TNFα occurred over a range of concentrations of Pam_3Csk_4 and for CpG up to 1 μM. Unlike its effect on TLR4, these results suggest that CD45 is a negative regulator of TLR2 and TLR9-mediated proinflammatory cytokine production.

3.2.6. CD45 is a positive regulator of IFNβ production

When CD45-/- BMDC are stimulated through TLRs that are dependent on MyD88 (TLR2 and TLR9), proinflammatory cytokine production is enhanced. TLR4 signaling uses both MyD88 dependent and independent pathways. This led us to hypothesize that CD45 could be negatively regulating MyD88 dependent signals leading to cytokine production (as is the case for TLR2 and TLR9) and positively impacting the MyD88 independent pathway present in TLR4 signaling, leading to a net neutral or positive effect. To further establish this hypothesis, we sought to examine IFNβ production, which is generated by a MyD88 independent pathway in both TLR3 and TLR4 signaling (75, 118). Figure 3.17A shows that lack of CD45 reduces IFNβ production in response to LPS, consistent with CD45 positively regulating this arm of the pathway. Interestingly, no IFNβ was made when low concentrations of LPS were used (10 ng/ml). To test this hypothesis further, IFNβ

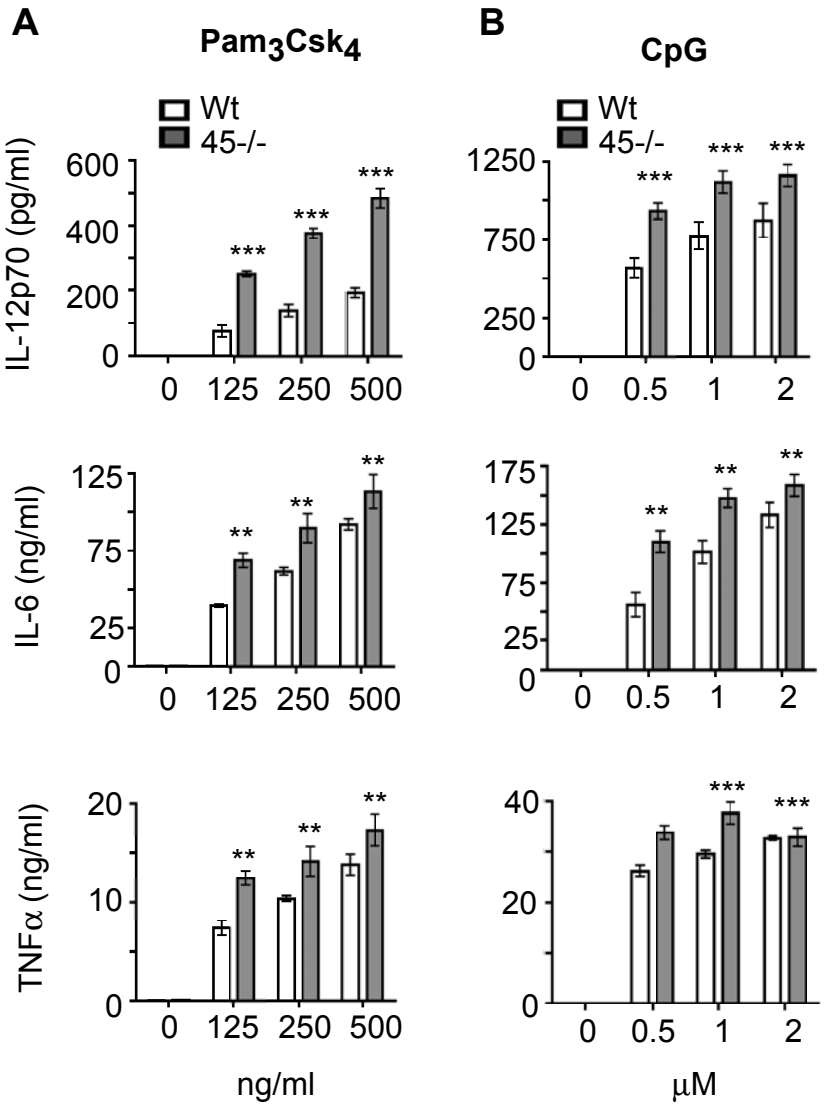


Fig 3.16 Proinflammatory cytokine from TLR2 and TLR9 activated CD45$^{+/+}$ and CD45$^{-/-}$ BMDC. Purified day 8 CD45$^{+/+}$ (Wt) and CD45$^{-/-}$ BMDC were stimulated with the indicated concentrations of (A) Pam_3Csk_4 or (B) CpG for 20-24 hours. The amounts of IL-12p70, TNFα, and IL-6 in the culture supernatant were determined by ELISA. Graphs are the mean ± SEM of three mice in an individual experiment. Experiments were repeated at least 3 times.

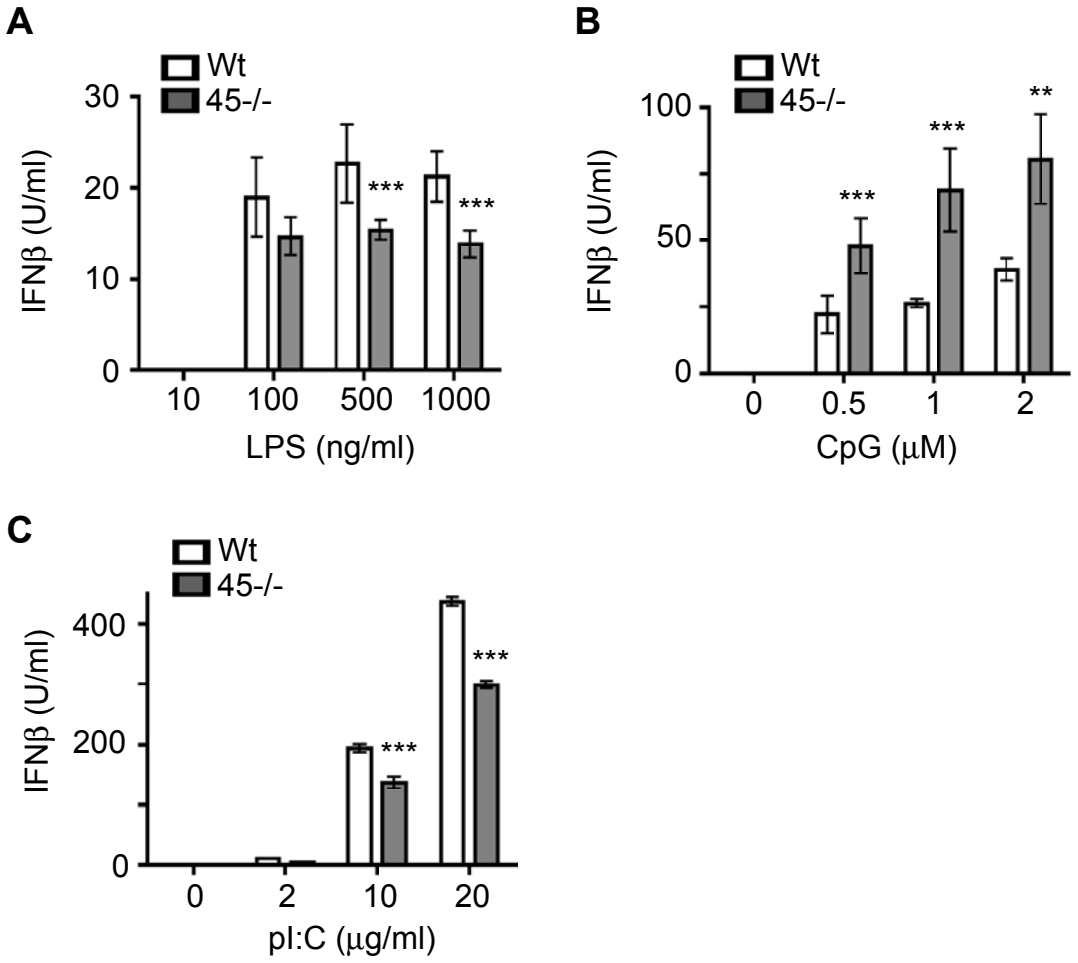


Fig 3.17 IFNβ secretion from CD45$^{+/+}$ and CD45$^{-/-}$ TLR activated BMDC. Purified day 8 CD45$^{+/+}$ (Wt) and CD45$^{-/-}$ BMDC were activated with the indicated concentrations of (A) Ultrapure LPS, (B) CpG or (C) poly (I:C) for 20-24 hours. The amount of IFNβ in the supernatant was quantified by ELISA. Graphs are the mean ± SEM from a representative experiment from three mice. Experiments were repeated 2-4 times. For all panels, ** $p < 0.01$, *** $p<0.001$.

production was measured after TLR9 and TLR3 receptor stimulation with CpG and polyriboinosinic polyribocytidylic (poly I:C) respectively as TLR9 signaling is all MyD88 dependent (119) whereas TLR3 signaling is MyD88 independent**.** Figure. 3.17B shows that CD45 null dendritic cells make more IFNβ upon CpG stimulation, consistent with a positive effect on MyD88 dependent signaling and less IFNβ with poly I:C (fig 3.17C), which signals via TLR3 in a MyD88 independent manner. This data supports the hypothesis that CD45 negatively regulates MyD88 dependent TLR signaling and positively regulates MyD88 independent TLR signaling.

3.3 Discussion

3.3.1 Data summary

Studies outlined in this chapter have demonstrated a role for CD45 in DC development and activation. In the spleen, DCs exist as heterogeneous populations that are functionally and phenotypically distinct. The absence of CD45 in mice leads to a skewing of splenic DC populations resulting in an accumulation of $CD8\alpha^+$ DCs and a concomitant decrease in the frequency of their $CD11b^+$ counterparts. In the case of GM-CSF-derived BMDC, which are a homogenous population of $CD11c^+CD11b^+$ myeloid cells, the lack of CD45 did not impair their *in vitro* development. These observations suggest that in the more complex *in vivo* environment, the loss of CD45 alters the development of DCs but not *in vitro* in response to GM-CSF signals.

Previous studies have identified a role for CD45 substrates, the Src-family of protein tyrosine kinases, in TLR-mediated cytokine secretion. During the course of this work, other investigations have demonstrated that the lack of CD45 in DCs leads to enhanced IL-6 and TNFα secretion in response to CpG and poly I:C stimulation (120). This chapter has extended this observation to include TLR2 ligands and has found an opposite effect using the TLR4 ligand, LPS. In addition, TLR-mediated IFNβ secretion is differentially affected by the loss of CD45, with the outcome determined by the TLR ligand used. Pathways strictly dependent on MyD88 showed an increase in both proinflammatory cytokine production and IFNβ secretion whereas in TLR3 and TLR4 signaling pathways, where MyD88 is dispensable, there was a decrease in IFNβ secretion. This led to the hypothesis that CD45 is a

negative regulator of MyD88 dependent TLR signaling and a positive regulator of MyD88 independent TLR signaling.

3.3.2 CD45 and dendritic cell development

We have shown that splenic DC can develop in the absence of CD45, but there is a skewing towards the $CD8\alpha^{+}CD11b^{-}$ population and a decrease in the $CD8\alpha^{-}$ $CD11b^{+}$ population in splenic $CD11c^{hi}$ dendritic cells. Given that the $CD8\alpha^{+}$ population is most efficient at cross-presentation and CD8 T cell activation, one would predict that if $CD45^{-/-}$ mice had functional T cells, they would be more efficient at these processes and less efficient at CD4 helper T cell responses, which are mediated by the $CD8\alpha^{-}$ dendritic cell subset (1).

Studies from long-term splenic stromal cultures (121), parabiotic studies (122) and observed DC precursor activity in non-DC areas of the spleen (123) have supported the idea that DCs are derived *in situ* in the lymphoid organs. In the $CD45^{-/-}$ mouse, the splenic composition is very different and thus the cytokine milieu required for DC development is likely altered. To determine this, DC development in a $CD45^{-/-}RAG2^{-/-}$ mouse was compared to that in a $CD45^{+/+}RAG2^{-/-}$ mouse. Since the DC subsets in the $CD45^{-/-}RAG^{-/-}$ spleens were still skewed towards the CD8α subset, this suggests that CD45 itself may contribute to the process of DC subset development. Given that certain subpopulations of DCs have different function and that $CD45RB^{hi}$ cells have been implicated in tolerance induction, the absence of CD45 could result in alterations in the balance of tolerance versus immunity.

The subset skewing observed in the absence of CD45 is in contrast with the $Lyn^{-/-}$ mouse where an accumulation of $CD11b^{+}$ dendritic cells occurred in the spleen, becoming

very pronounced as the mice aged. However, the case of $CD45^{-/-}$ cells is somewhat more complex than the simple knockout of Lyn. CD45 regulates not just Lyn but also Fyn and Hck (see chapter 4). Loss of CD45 dysregulates all of the above Src-family members that may play different roles in cell development. Adding to the complexity, CD45 can dephosphorylate both the positive and negative regulatory sites of SFKs and the balance of phosphorylation on these sites may then determine if loss of CD45 leads to an over-active or inactive kinase.

Interestingly, $CD45^{-/-}$ mice contain a higher number of splenic pDC. Although the frequency of pDC in the $CD45^{-/-}$ mice is not significantly different from the frequency of pDC in the C57BL/6 mice, the $CD45^{-/-}$ spleen has approximately 2-fold more cells. This leads to a tendency for the $CD45^{-/-}$ mice to have numerically more pDC (data not shown). However, with the high experimental variability, this difference was not statistically significant. This data is in agreement with another study by Montoya *et al*. (110). The increase in pDC numbers was more obvious when the CD45 knockout was crossed onto the RAG2-knockout background and compared to the $CD45^{+/+}$ RAG2 knockout. Unlike other DCs, it is believed that pDC develop in the bone marrow (BM) or the blood and traffic to lymphoid organs (124). The increase in pDC in $CD45^{-/-}$ spleens may reflect an increase in their mobilization from the BM, an increase in their generation from progenitors, a decrease in their turnover or even increased retention in the spleen. Since CD45 has been shown to regulate integrin signaling, cytokine signaling, chemokine signaling (125) and apoptosis, it would be of interest to determine the role of CD45 in pDC functions.

Unlike splenic DCs, the loss of CD45 during *in vitro,* GM-CSF-driven DC differentiation from BM had no substantial effect. The loss of CD45 in BMDC also leads to a

different phenotype than that reported in BMDC cultures from $Lyn^{-/-}$ mice. $CD45^{-/-}$ BMDC accumulated in the non-adherent fraction of the culture at roughly the same rate and to the same extent as in the wild-type cultures. $Lyn^{-/-}$ BMDC expanded to higher numbers than their wild-type counterparts although by day 6 of culture, the $Lyn^{-/-}$ $CD11c^{+}$ cells were present at a similar frequency as the wild type. This data suggests that in the absence of Lyn, more myeloid cells accumulate but that DCs are generated in the same proportion, an effect similar to that observed in the $CD45^{-/-}$ cultures.

3.3.3 CD45 in DC maturation

One hallmark of DC maturation is the expression of high levels of costimulatory molecules on the cell surface to drive T cell activation. Under steady-state conditions in the periphery, where no engagement of the pattern-recognition receptors has occurred, these molecules are expressed at low levels on DCs to prevent activation of T cells by DCs bearing self-antigens. Under these conditions, the DC has classically been described as "immature". Immature DC in the periphery undergo a partial maturation process where they upregulate the chemokine receptor CCR7 and migrate to the lymph nodes. These semi-mature DCs have been found to phenotypically resemble activated DCs (which have encountered a pathogenic stimulus) in their upregulation of costimulatory molecules (126), yet these DCs induce T cell tolerance. The process of pathogen recognition by a DC converts it to an immunogenic state where, in addition to expressing costimulatory molecules, it will express higher levels of MHC-antigen complexes and secrete proinflammatory cytokines (127).

In the absence of an activation signal, the loss of CD45 led to a slight increase in CD40, CD80 and CD86 co-stimulatory molecule expression in splenic and BMDC. This increase persisted after upregulation of the co-stimulatory molecules by LPS but was not observed consistently in CpG, poly I:C or Pam_3Csk_4 stimulated BMDC. Higher levels of expression of CD40, CD80 and MHCII were observed by Piercy *et al.* (120) in unstimulated $CD45^{-/-}$ BMDC and also after CpG and poly I:C stimulation. A possible explanation for the discrepancy may relate to the fact that Piercy *et al.* used a 5-fold higher concentration of poly I:C than was used in this study and also a different CpG sequence (1828 versus 1668). This higher level of expression of co-stimulatory molecules prior to stimulation suggests that CD45 has a slight negative regulatory effect on co-stimulatory molecule expression, possibly resulting from a slight enhancement of spontaneous dendritic cell maturation in the absence of pathogenic stimuli. This effect is noticeably different from that observed in the $Lyn^{-/-}$ mice where co-stimulatory molecule expression is decreased prior to stimulation and upon LPS stimulation (107, 128). Once again, this may be due to the fact that multiple SFKs, and any other proposed CD45 substrates like the Janus kinases, will be affected by the absence of CD45.

The signaling pathways leading to costimulatory molecule upregulation in DCs are not fully defined. The upregulation of costimulatory molecule expression appears to involve different adaptors and signaling components for the different TLRs. For LPS, the Trif-Tram pathway seems to play an important role but for TLR3, which only uses Trif for cytokine secretion, there is also a Trif-independent pathway for increasing costimulatory molecule expression (129). CpG-driven cytokine secretion is strictly dependent on MyD88 and, at least in the case of CD40, costimulatory molecule expression does also (130). TRAF6, which is

central to all TLR signaling pathways, is also crucial for expression of high levels of costimulatory molecules (131) upon TLR ligation. Without having the signaling pathways leading to costimulatory molecule upregulation for the different TLRs fully defined, at this juncture it is difficult to speculate on where CD45 and the SFKs may be acting in this process.

3.3.4 CD45 and TLR-mediated cytokine secretion

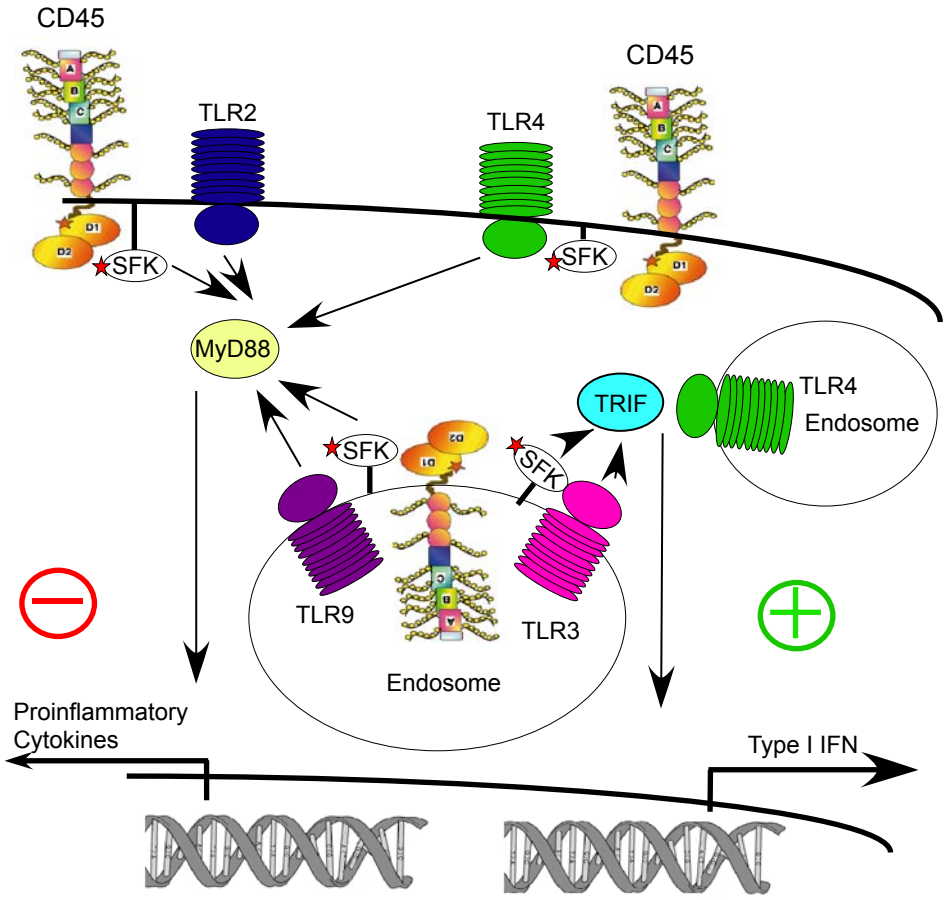
The production of proinflammatory cytokines by TLR activated DCs is vital for driving T cell activation and polarization of the immune response may be critical for overcoming Treg-mediated suppression (132). DCs express a variety of TLRs that are capable of recognizing a large variety of pathogens. Since past studies have identified a potential role for SFKs in TLR-driven cytokine production, it was not surprising that the loss of CD45 in DCs affected the production of IL-12, IL-6 and TNFα. What was intriguing was that the ligation of the different TLRs had different results.

A major finding from this work is that TLR induced proinflammatory cytokine secretion and IFNβ production is regulated by CD45. Both positive and negative effects of CD45 were observed. CD45 had a negative effect on TLR stimulation via a MyD88 dependent pathway (TLR2 and TLR9) and a positive effect on TLR induced IFNβ production via a MyD88 independent pathway (TLR3). The differential use of MyD88 dependent and independent pathways by different TLRs provides one plausible explanation for the observed positive and negative effects of CD45 on specific TLR activated cytokine production. TLR3 signaling is entirely MyD88 independent and CD45 has a clear positive effect on IFNβ

production in response to poly I:C. Unlike Piercy *et al*., (120) no proinflammatory cytokines were detected after stimulation with poly I:C (again, this may be due to the dose used).

The negative effects of CD45 on TLR2 and 9 signaling are consistent with their exclusive dependence on the MyD88 pathway for proinflammatory cytokine production. Increases in TLR9 driven proinflammatory cytokine production were also observed in the Piercy paper. This negative effect of CD45 is also seen in TLR9 signaling leading to IFNβ production, which in this case is also mediated via a MyD88 dependent pathway. Thus, there is a good correlation between the effect of CD45 and the pathways used by the different TLRs. Since TLR4 uses both MyD88 dependent and independent signaling pathways, the net effect of CD45 on LPS induced cytokine production is predicted to be a product of its negative and positive effects on the MyD88 dependent and independent pathways respectively. With 10 ng/ml of LPS, no detectable IFNβ is produced, negating any positive effect of CD45 on the MyD88 independent pathway thereby allowing the negative effect of CD45 on the MyD88 pathway to predominate. At higher levels of LPS, IFNβ is produced, allowing the positive effect of CD45 to come into play and contribute to an overall net positive effect at 100 ng/ml LPS and to a balanced net effect at 1 μg/ml LPS. Thus, the overall effect of CD45 on LPS induced proinflammatory cytokine production appears to be a product of its negative effect on the MyD88 pathway and its positive effect on the MyD88 independent, IFNβ pathway. While proinflammatory cytokine production has primarily been associated with the MyD88 dependent TLR signaling pathway, a recent report from Gautier *et al*., indicates that secreted IFNβ activates a feedback loop by binding to the type I IFN receptor on the dendritic cell, which not only stimulates more IFNβ secretion, but also enhances IL-12p70 secretion (133). This helps to explain how an effect of CD45 on the

MyD88 independent pathway leading to IFNβ production can impact proinflammatory cytokine secretion via the MyD88 dependent pathway and the model is depicted in figure 3.18. The next chapter will explore the TLR signaling pathways in an attempt to identify a mechanism by which CD45 exerts its effects.



3.18 Proposed model for the action of CD45 in the MyD88-dependent and independent pathways of TLR signaling.

Chapter 4 Determining how TLR signaling events are affected by lack of CD45

4.1 Introduction

In the previous chapter, the absence of CD45 was found to have a differential effect on proinflammatory cytokine secretion dependent upon the TLR stimulated. The aim of this chapter was to determine the mechanism by which the absence of CD45 affects TLR signaling leading to differences in proinflammatory cytokine secretion. Based on the evidence presented in the previous chapter, we hypothesized that CD45 is a negative regulator of MyD88-dependent signaling (evident with TLR2 and TLR9 stimulation) and a positive regulator of the MyD88-independent pathway (observable in the decreased IFNβ release after TLR3 or TLR4 stimulation). The study was initiated by looking initially at the total tyrosine phosphorylation pattern in unstimulated BMDC. Since CD45 is a tyrosine phosphatase we expected that in $CD45^{-/-}$ DCs, direct substrates would be hyperphosphorylated whereas tyrosine kinase substrates would likely be hypophosphorylated. The tyrosine phosphorylation state and expression patterns of the SFK themselves was also examined. Secondly, activation of molecules involved directly downstream of the TLR was examined to determine if the absence of CD45 had a direct effect on TLR signaling. Lastly, the phosphorylation of negative regulators of TLR-mediated proinflammatory cytokine secretion was examined following LPS stimulation.

4.2 Results

4.2.1 Unactivated CD45$^{-/-}$ BMDCs have altered phosphotyrosine patterns, but no observable differences upon LPS stimulation.

In NK cells devoid of CD45, alterations in the basal phosphotyrosine levels occur (134). Therefore it was of interest to determine if this was also the case in BMDCs. BMDCs from day 8 cultures were harvested and lysed prior to SDS-PAGE and western blotting with 4G10 to examine phosphotyrosine patterns. Alterations in cellular phosphotyrosine levels from lysates of CD45$^{-/-}$ BMDCs were observed (fig 4.1a), in particular at bands around 130 and 66 kDa. The 130 kDa band was hyperphosphorylated in the CD45$^{-/-}$ DC lysate, indicative of a potential CD45 substrate, whereas the 66 kDa band was hypophosphorylated, suggesting it is a substrate of a tyrosine kinase regulated by CD45. Since CD45 has been implicated in Jak kinase regulation (81) and Jak kinases have a molecular weight of 130 kDa, Jak1 and 2 kinases were immunoprecipitated from unstimulated day 8 BMDC. However, they were not tyrosine phosphorylated (Fig. 4.1b). Another potential candidate was the inositol polyphosphate 5'-phosphatase (SHIP). This was found to be hyperphosphorylated in CD45$^{-/-}$ BMDC (Fig. 4.1c), but it was not the p130 protein. It is likely that isolation from the gel and mass-spectrometry sequencing will be required to determine the identity of these proteins.

Since two bands in the unstimulated CD45$^{-/-}$ BMDC lysate showed altered phosphotyrosine patterns when unstimulated, analysis of tyrosine phosphorylation in LPS

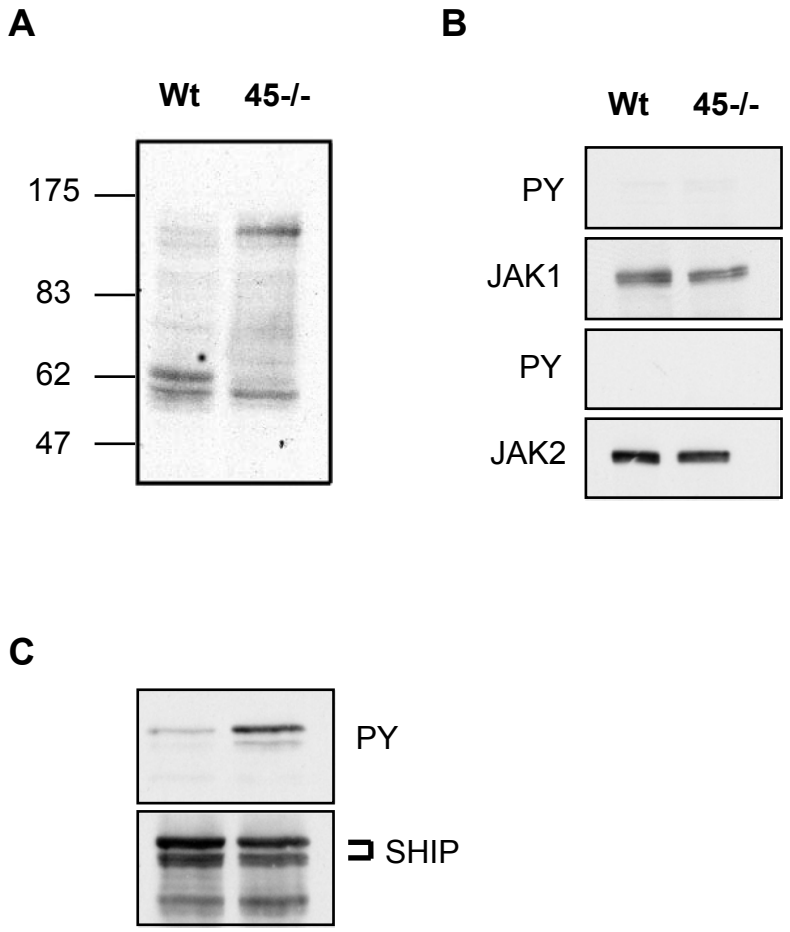


Fig 4.1 Tyrosine phosphorylation is altered in CD45$^{-/-}$ unstimulated BMDC. *A*, Basal tyrosine phosphorylation in whole cell lysate. BMDC were lysed in 1% Tx-TNE and phosphotyrosine assessed by immunoblotting with 4G10. Blot is one representative of 3 experiments. *B*, Jak phosphorylation in day 8 BMDC. BMDC were lysed and Jak 1or 2 was immunoprecipitated. Phosphotyrosine and Jak loading were determined by immunoblotting. The blot is representative of 3 experiments. *C*, Phosphorylation of SHIP in day 8 BMDC. SHIP1 was immunoprecipitated from day 8 BMDC and loading and phosphotyrosine levels were determined by immunoblotting. Blot is one representative of at least three experiments.

stimulated BMDC lysates was examined. Unlike unstimulated BMDC, $CD45^{-/-}$ LPS-activated BMDC did not show any significant differences in gross tyrosine phosphorylation (Fig 4.2).

4.2.2 Altered tyrosine phosphorylation of Src-family kinase members in $CD45^{-/-}$ BMDCs.

Since the major substrates for CD45 in lymphocytes and macrophages are members of the Src family of tyrosine kinases, which migrate between 50-60 kDa, we next evaluated the phosphorylation status of Src family kinases in BMDC. Src family kinases were immunoprecipitated from unstimulated DC lysates and their tyrosine phosphorylation determined by blotting with the anti-phosphotyrosine mAb, 4G10. Figure 4.3a shows the presence of Hck, Lyn, Fyn, Src and Fgr in BMDC. Only Lyn, Hck and Fyn were phosphorylated in the $CD45^{+/+}$ cells and hyperphosphorylated in $CD45^{-/-}$ DC lysates. Fig. 4.3b indicates the extent of hyperphosphorylation for Fyn, Hck, and Lyn over three independent experiments. The increased tyrosine phosphorylation of Lyn, Hck and Fyn suggests that their functional activities may be dysregulated in $CD45^{-/-}$ dendritic cells. Although not well defined, Lyn has been implicated in TLR4 signaling in DCs leading to the upregulation of co-stimulatory molecule expression and proinflammatory cytokine production (107, 128).

To determine if the lack of CD45 affects the ability of Lyn to participate in TLR4 signaling, we examined the activation and phosphorylation status of Lyn upon stimulation with 100 ng/ml of Ultrapure LPS. In unstimulated $CD45^{-/-}$ BMDC, Lyn is hyperphosphorylated (4.4a), but not at the autophosphorylation site (Fig. 4.4b). Upon LPS

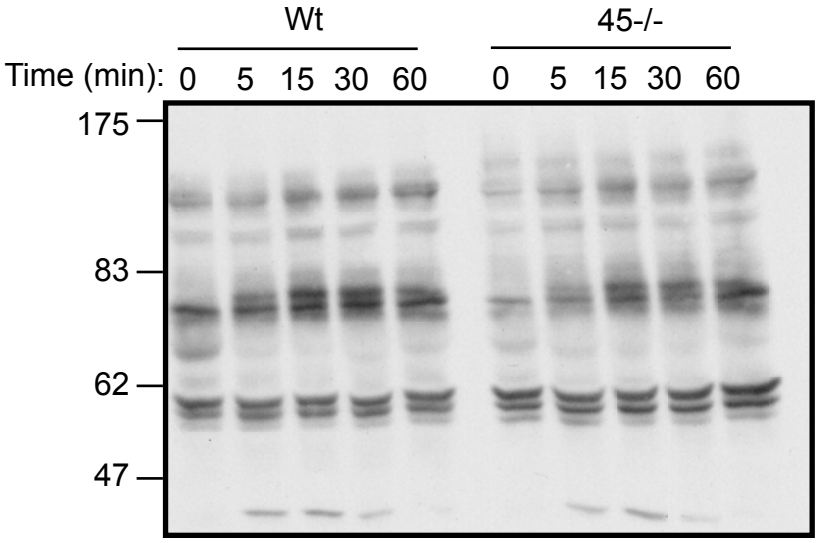


Fig 4.2 Phosphotyrosine patterns in the lysates of LPS stimulated BMDC. $CD45^{+/+}$ (Wt) and $CD45^{-/-}$ (45-/-) BMDC were stimulated with 1ug/ml of Sigma LPS for the time points indicated prior to lysis and SDS-PAGE. Phosphotyrosine was visualized by western blot using 4G10. The blot is one representative of three independent experiments.

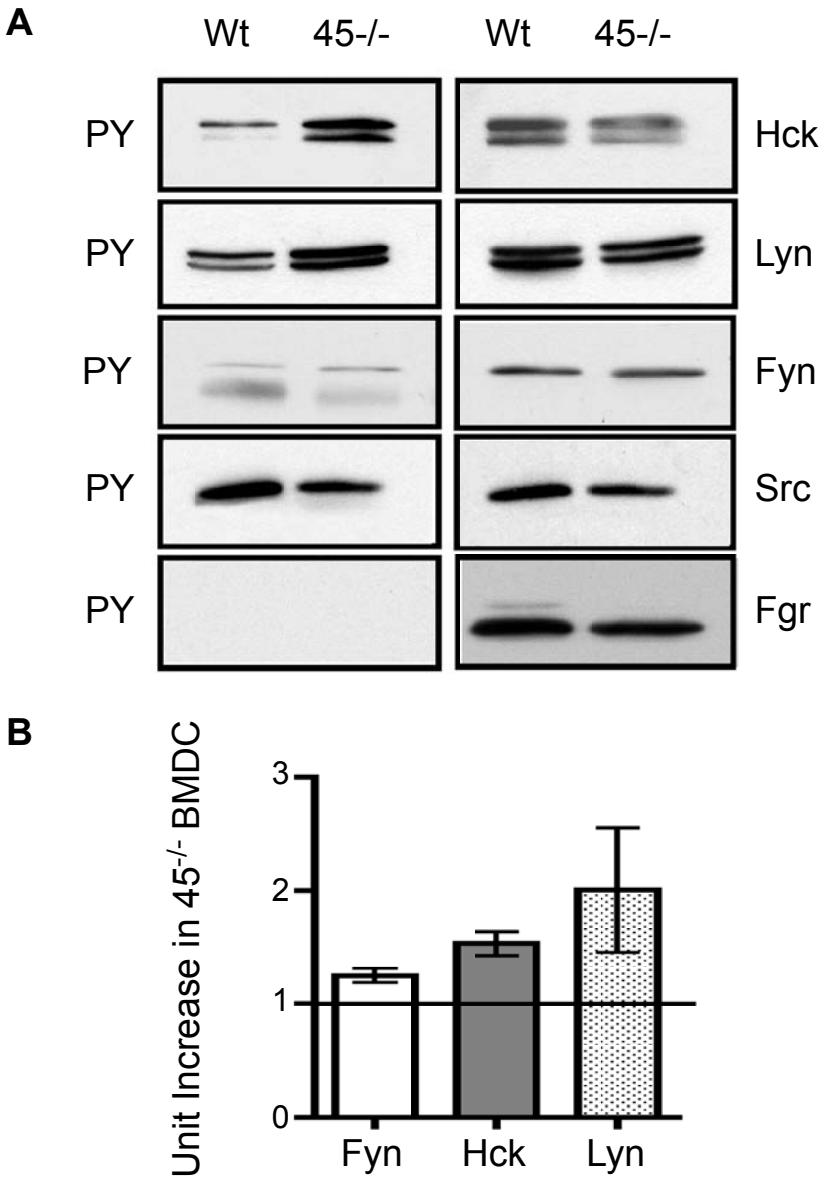


Fig 4.3 Src family kinase phosphorylation and expression in BMDC. (A) Various Src family kinases were immunoprecipitated from day 8 BMDC and loading and phosphotyrosine levels were determined by immunoblotting. The left panel shows the phosphotyrosine blots and the right hand panel is the loading blot. One representative experiment of three is shown. (B) Densitometric analysis of the average fold increase (± SEM) in Src family kinase phosphorylation in $CD45^{-/-}$ BMDC over three experiments. Integrated density values of the phosphotyrosine level were divided by those obtained for the loading control, the value for $CD45^{+/+}$ was then set to 1.

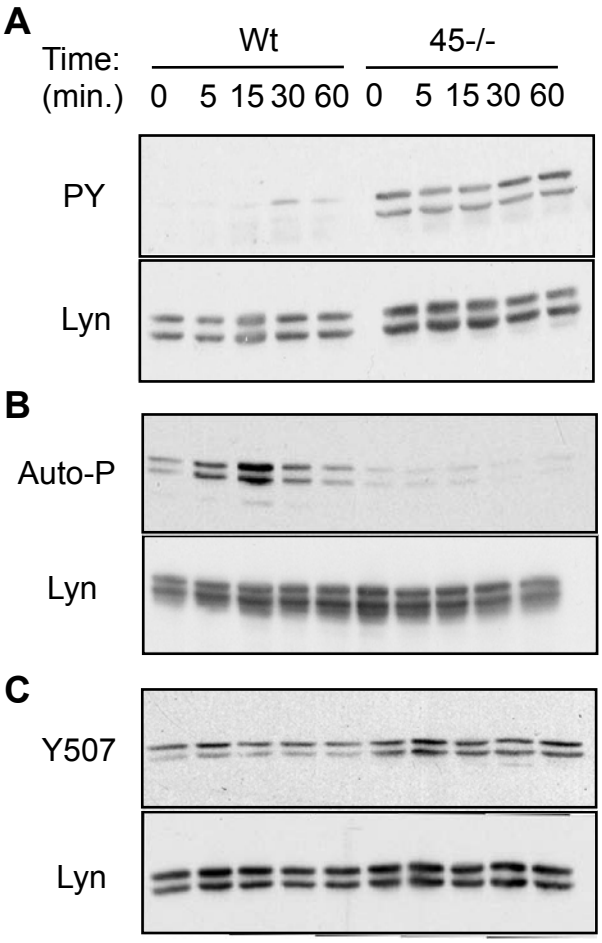


Fig 4.4 Activation of Lyn in BMDC upon LPS stimulation. Lyn activation was assessed by Lyn immunoprecipitation from BMDC stimulated with 100 ng/ml of LPS for the indicated times prior to SDS-PAGE. Phosphorylation at the activation site (Auto-P) was determined using antisera to phospho-Src416 that cross-reacts with the corresponding activation site on Lyn (Y396), phosphorylation at the inhibitory tyrosine was determined by using an antibody specific to LynY507. Detection was done first for phosphotyrosine (Y416 or Y507 or total PY) and then stripped and re-probed for Lyn. One representative from three experiments is shown.

stimulation, Lyn was inducibly phosphorylated at the autophosphorylation site in $CD45^{+/+}$ DCs, whereas no comparable induction was seen in $CD45^{-/-}$ DCs (Fig. 4.4b) and an increase in phosphorylation at the negative regulatory site was observed (Fig 4.4c). This suggests that Lyn is hyperphosphorylated at the negative regulatory site in the absence of CD45 and is not inducibly phosphorylated or activated upon LPS stimulation.

4.2.3 No effect of the lack of CD45 on canonical TLR signaling molecules in either TLR2 or TLR4 signaling

To examine possible downstream effects of altered Lyn activation in TLR4 signaling, the induction of MAPK phosphorylation upon LPS stimulation was investigated. Figure 4.5a shows no obvious differences in the phosphorylation status of Erk, Jnk or p38, which were all phosphorylated with similar kinetics and intensities in both $CD45^{+/+}$ and $CD45^{-/-}$ BMDC. Since SHIP phosphorylation was altered in unstimulated BMDC, we examined the possibility that the PI3 kinase pathway may be dysregulated in $CD45^{-/-}$ BMDC. Like the Src family kinases, the involvement of PI3K pathway in LPS signaling is not well understood with both positive and negative effects being reported (135). However, analysis of phospho-Ser 473 of Akt did not reveal any major differences between $CD45^{+/+}$ and $CD45^{-/-}$ BMDC (Fig. 4.5a).

Since LPS induced cytokine production requires the MyD88 dependent signaling pathway leading to NF-κB activation, we assessed whether NF-κB activation was affected. SDS-PAGE analysis of the translocation of NF-κB p65 into the nucleus as well as its ability to bind DNA, assessed by ELISA, revealed no observable differences in the kinetics or levels of translocated NF-κB or its DNA binding activity (Fig. 4.5b and c). Thus, no obvious defect

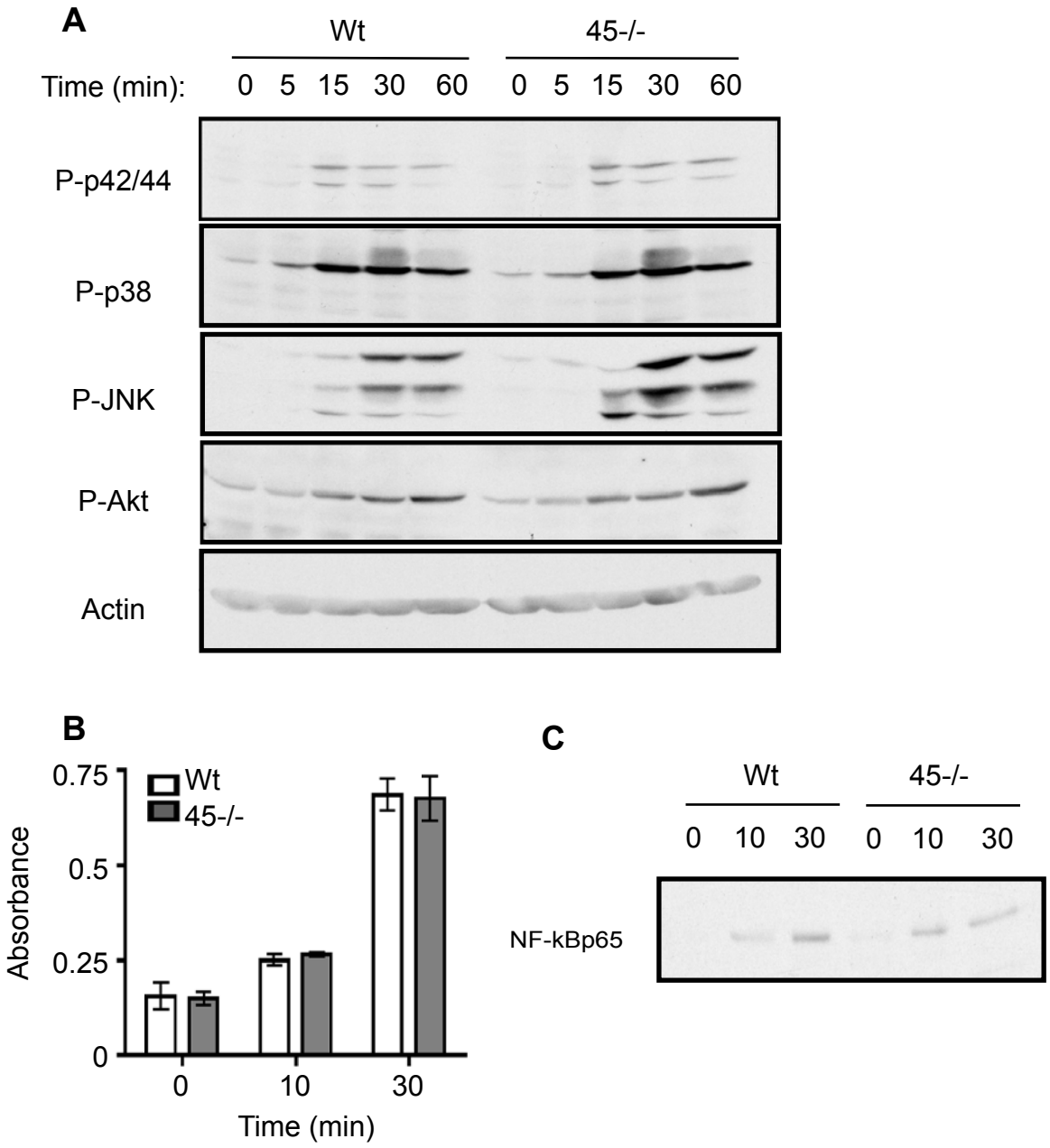


Fig 4.5 Activation of canonical TLR4 signaling molecules in the presence and absence of CD45. (A) Phosphorylation of various signaling molecules in response to 100 ng/ml of Ultrapure LPS was assessed by separation of cell lysates by SDS-PAGE and immunoblotting with phospho-specific antibodies. Loading was determined by blotting for actin. For activation of NF-κB p65, pooled BMDC from 3 mice were activated with 100 ng/ml of Ultrapure LPS for the indicated times and then lysed and left unfractionated for ELISA (B) or the nuclear fraction isolated and separated on SDS-PAGE and immunoblotted for NF-κB p65 (C). Experiment is one representative of 3.

was found in the canonical MyD88 dependent signaling pathway directly downstream of TLR4 in $CD45^{-/-}$ DCs.

In the event that feedback from the MyD88-independent pathway in LPS signaling was confusing the signals, the activation of Erk, Jnk and p38 were also examined after TLR2 stimulation (Fig 4.6). Once again, no clear differences in the activation of these molecules were observed in $CD45^{-/-}$ BMDCs.

4.2.4 Lyn substrates and negative regulators of TLR signaling are not activated upon TLR4 stimulation of $CD45^{-/-}$ BMDC.

No direct effect on signaling downstream of either TLR4 or TLR2 was observed in $CD45^{-/-}$ BMDC but the activation of Lyn was impaired upon LPS stimulation (Fig 4.4). Therefore, the next step taken was to look at the activation of Lyn substrates upon TLR4 stimulation. Dok1 and Dok2 are cytoplasmic adaptor proteins that recruit SH2-domain containing molecules like Nck and RasGAP and have been identified as substrates for tyrosine kinases (136, 137). In B cell antigen receptor signaling, Lyn is required for the phosphorylation of Dok1 (138) and Dok1 and Dok2 were recently discovered to be negative regulators of TLR4-mediated cytokine secretion (139). To determine if the impaired activation of Lyn upon TLR4 stimulation might also alter the phosphorylation of Dok2, day 8 purified BMDC were stimulated with LPS and Dok2 was immunoprecipitated. Figure 4.7a shows that Dok2 is not inducibly phosphorylated in $CD45^{-/-}$ BMDCs upon LPS stimulation although it is strongly tyrosine phosphorylated in $CD45^{+/+}$ BMDCs.

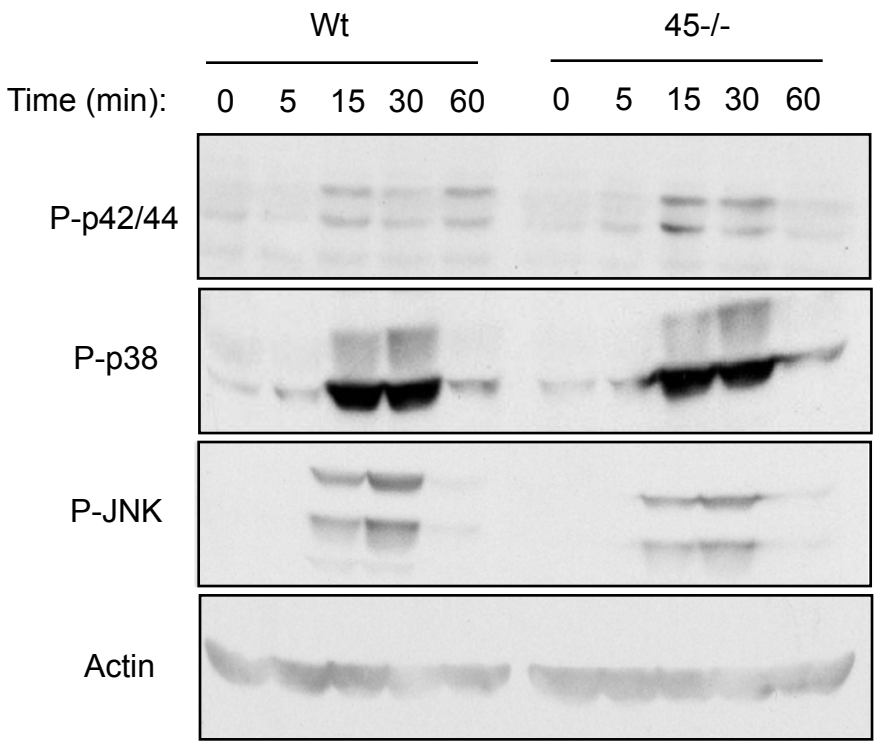


Fig 4.6 Activation of TLR2 signaling molecules in CD45$^{+/+}$ and CD45$^{-/-}$ BMDC.
1X10^{6} purified, day 8 BMDCs from CD45$^{+/+}$ (Wt) and CD45$^{-/-}$ cultures were stimulated with 125ng/ml of Pam_3Csk_4 prior to lysis at the time points indicated. Lysates cleared of debris and the cytosolic fraction was separated by SDS-PAGE and transferred to PVDF for western blotting. Blots were probed for activated Erk, JNK and p38 and actin was used to determine equal loading.

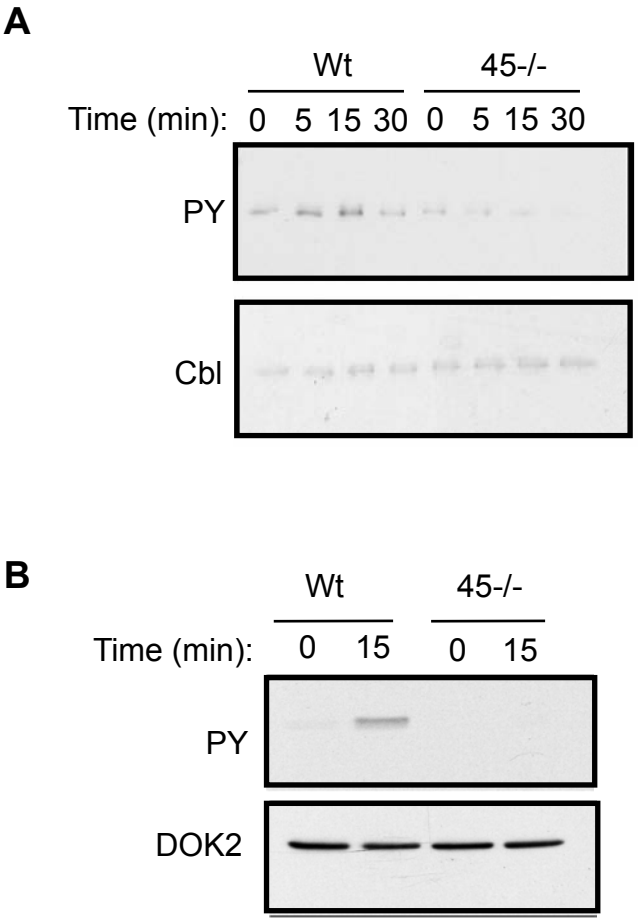


Fig 4.7 Negative regulators of the MyD88-dependent pathway are not activated in LPS-stimulated CD45$^{-/-}$ BMDC. Activation of Cblb and DOK2 in BMDC were determined by immunoprecipitation of either (A) Cblb or (B) DOK2 upon stimulation with LPS for the timepoints indicated. Detection of phosphotyrosine (4G10) was done first and then the blots were stripped and re-probed for protein loading. Experiments are 1 representative of 4 independent experiments.

Another SFK substrate is the E3 ubiquitin ligase, Cbl. Cbl associates with Lyn in a variety of signaling pathways (140, 141) and loss of Cbl increases proinflammatory cytokine secretion in mouse models of sepsis (142). Cblb was also recently identified as a negative regulator of TLR4-mediated cytokine release. Cblb association with TLR4 and MyD88 places it in the MyD88-dependent pathway. It was of interest to determine if the SFK dysregulation observed in $CD45^{-/-}$ BMDC would change the phosphorylation status of Cbl upon TLR4 stimulation. Purified BMDC were activated with 100ng/ml of UltraPure LPS for the time points indicated in figure 4.7b and Cbl was immunoprecipitated for analysis by western blotting. Cbl phosphorylation was not detected in $CD45^{-/-}$ BMDC. Although not definitive, the lack of activation of negative regulators of TLR signaling may provide some evidence that through SFK family members, such as Lyn, CD45 can be a negative regulator of MyD88-dependent signaling.

4.2.5 TLR3 is hyperphosphorylated prior to stimulation in $CD45^{-/-}$ BMDC

The placement of the SFK in TLR signaling pathways is not clear and although the consensus in the literature is that SFK are activated upon TLR ligation (49), their role in the downstream signaling process is unclear with different approaches yielding disparate results. The triple knockout of Hck/Fgr/Lyn in mouse macrophages showed no significant defect in cytokine secretion or signaling in macrophages stimulated with LPS/IFNγ (148). The double Hck/Fgr knockout mice were resistant to endotoxic shock (143) and Lyn deficient DCs also show impaired cytokine secretion with LPS stimulation. Additionally, the use of the SFK

inhibitors PP1 and PP2 have been documented to decrease cytokine secretion upon both TLR4 (113, 144) and TLR9-mediated cytokine secretion (112, 145).

More recently, data has been published showing that TLR tyrosine phosphorylation is important for downstream signaling. Tyrosine phosphorylation has been detected on TLR2 (146), TLR3 (147),TLR4 (148, 149), TLR5 (150) and TLR9 (145) and can be abrogated with PP2 treatment (145, 148) suggesting a role for SFK in this process. Since $CD45^{-/-}$ BMDCs show altered SFK phosphorylation patterns, it was of interest to determine what effect this might have on TLR phosphorylation and if this could contribute to the changes observed in cytokine secretion. TLR antibodies that work well are very difficult to find. Because of the limited efficacy of the available reagents, TLR3 was chosen as a model because several other groups had been able to successfully immunoprecipitate it from murine cells using a commercially available antibody.

Phosphorylation of TLR3 has been demonstrated to result in activation of TBK1 and phosphorylation of IRF3 leading to type I interferon production (147). It was initially hypothesized that TLR3 would not be phosphorylated upon stimulation with poly I:C in $CD45^{-/-}$ BMDC. Surprisingly, TLR3 was hyperphosphorylated prior to stimulation (Fig 4.8a) and once the observed phosphotyrosine levels were adjusted for protein loading through densitometric analysis, there was approximately a 10-fold difference in the tyrosine phosphorylation on TLR3 in $CD45^{-/-}$ BMDC at earlier time points (fig 4.8b). At later time points (30 to 60 minutes) the levels were comparable to those observed in $CD45^{+/+}$ BMDC.

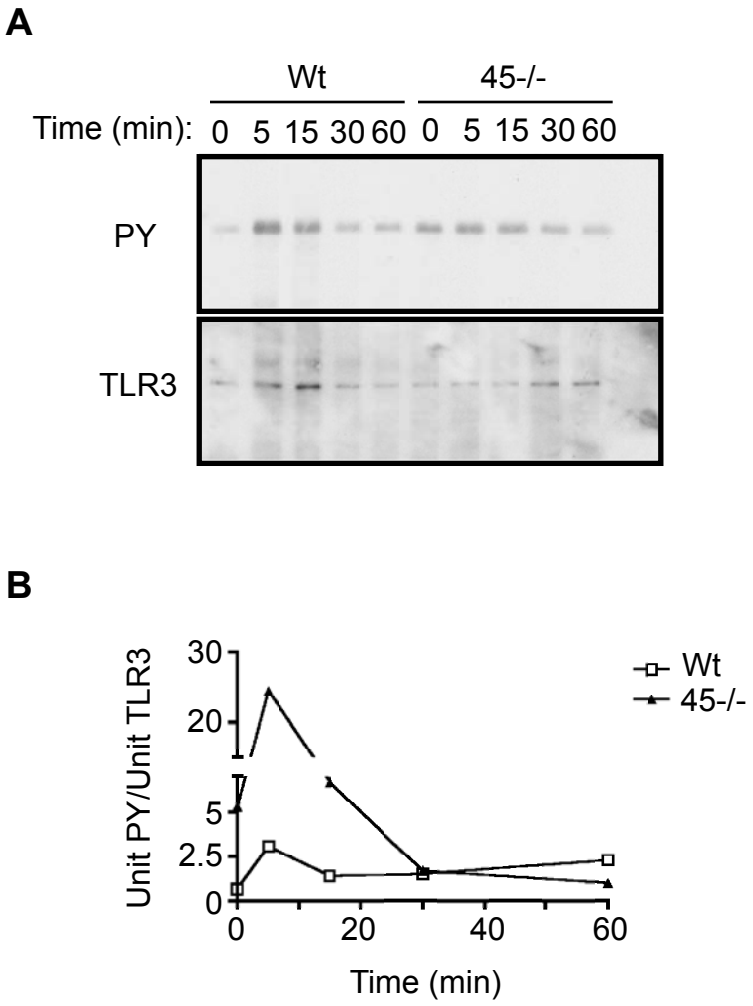


Fig 4.8 TLR3 is hyperphosphorylated in CD45$^{-/-}$ BMDC. Phosphorylation of TLR3 was determined by immunoprecipitation following stimulation with 20μg/ml poly (I:C) at the time points indicated. (A) Phosphorylation was assessed first and then the blot was stripped and re-probed for TLR3 loading. (B) Densitometric analysis to determine the relative degree of phosphorylation per amount of protein loaded. Integrated densitometry values for phosphotyrosine bands were divided by the integrated densitometry value obtained for the corresponding TLR3 loading band. The blot is representative of three independent experiments and the densitometry graph corresponds to the blot in the upper panel.

4.3 Discussion

4.3.1 Results summary

Alterations to in the phosphorylation status of CD45 substrates and SFK substrates were observed in both unstimulated and stimulated BMDCs. In unstimulated BMDC, unidentified proteins of approximately 130 kDa and 66 kDa showed altered tyrosine phosphorylation along with the SH2-containing 5'-inositol phosphatase, SHIP1. Although the identity of the 130 and 66 kDa proteins remains a mystery and the contribution of hyperphosphorylated SHIP to TLR signaling in the absence of CD45 remains to be defined, these results do show that the absence of CD45 causes changes in the resting state of BMDC and thus may be contributing to their ability to signal normally upon TLR ligation. Although no aberrations in gross phosphotyrosine patterns were visible on a western blot of crude lysates from LPS stimulated $CD45^{-/-}$ BMDCs, it is possible that affected proteins may not be present in sufficient quantities for detection by Western blot and thus elude observation using this approach.

The SFK can run close to 66 kDa on western blots and since they are identified substrates of CD45, their expression and basal phosphorylation in BMDCs was determined by immunoprecipitation and western blotting. Lyn, Hck, Fgr, Fyn and Src were all detectable in BMDCs but Lck was not (data not shown). There have been no previous reports of the presence of Fyn in BMDC so this is a novel finding. No phosphotyrosine was detected on Src or Fgr although quite a strong band was detected on the loading blot. In contrast, Lyn, Hck

and Fyn showed varying levels of hyperphosphorylation in the $CD45^{-/-}$ cells up to approximately 2-fold for Lyn.

Lyn is the SFK most commonly linked to TLR signaling and can be co-immunoprecipitated with TLR4 (151). For the next series of experiments, the ability of Lyn to be tyrosine phosphorylated upon TLR4 ligation was examined. In $CD45^{-/-}$ BMDCs, Lyn remained hyperphosphorylated after LPS addition and the site of phosphorylation was determined to be on the inhibitory residue Y507. The lack of phosphorylation on the auto-phosphorylation site suggests that Lyn is not activated upon LPS stimulation in the absence of CD45.

The role of SFK in TLR signaling remains unclear. However, the addition of PP2 to human monocyte derived macrophages found no effect on p38, JNK, p42/44 or NF-κB activation (144) and similar findings were obtained in macrophages from the Hck/Fgr/Lyn knockout. Nonetheless, it remained a possibility that the absence of CD45 could affect more than just the SFK so the activation of p42/44, p38, JNK and NF-κB via TLR4 were examined with no discernable difference detected between CD45-/- and Wt BMDC.

Differences were observed in the activation of negative regulators of TLR-mediated cytokine secretion, namely DOK2 and Cbl. These molecules were chosen for investigation because of their association with SFK in the literature and their involvement in proinflammatory cytokine secretion in TLR signaling pathways. Unlike the Wt, both Cbl and Dok2 were not robustly tyrosine phosphorylated in $CD45^{-/-}$ BMDC suggesting the possibility that the observed increases in MyD88-dependent cytokine secretion in these cells might be in part due to abridged activation of negative regulators. Another possibility is that CD45 itself may be able to dephosphorylate some of the tyrosine residues on the TLR itself that are

required to abbreviate signaling leading to proinflammatory cytokine secretion. TLR3 was observed to be hyperphosphorylated prior to stimulation in $CD45^{-/-}$ BMDC. Since TLR3 did not produce proinflammatory cytokines in this system, it may be that Lyn activity is required for phosphorylation of the TLR tyrosine residues upstream in the signaling cascade leading to IRF3 activation.

4.3.2 Dysregulation of Src-family kinases $CD45^{-/-}$ BMDC

Investigation into the molecular effects of the absence of CD45 revealed the hyperphosphorylation of Hck, Lyn, Fyn and SHIP in GM-CSF derived BMDC. LPS stimulation induced transient Lyn activation in the $CD45^{+/+}$ bone marrow cells but not in the $CD45^{-/-}$ cells, showing dysregulation of Lyn in the absence of CD45. However, further analysis of the phosphorylation states of MAPKs and Akt, as well as NF-κB p65 translocation and activation, did not reveal any major differences between LPS activated $CD45^{+/+}$ and $CD45^{-/-}$ BMDC. Both Lyn and PI3K are activated upon LPS stimulation of human monocytes (152) and treatment of human monocyte derived DCs with a Src family kinase inhibitor, PP1 decreased TNFα, IL6 and IL12p40 production in response to LPS, indicating a role for Src family kinase activity in proinflammatory cytokine secretion (113). Analysis of BMDC from $Lyn^{-/-}$ mice also showed a positive role for Lyn, as reduced levels of proinflammatory cytokines were produced in response to 1-4 ng/ml LPS and CpG (107). In contrast to this, macrophages from Lyn, Hck and Fgr triple knockout mice show normal or slightly enhanced cytokine responses to LPS and IFNγ (153), suggesting a dispensable role for, or interplay between, Src family kinases in LPS signaling. Recent data indicates that

Hck and Lyn are activated in response to CpG in a TLR9 independent manner, yet are required for the subsequent TLR9-MyD88 dependent cytokine production in a human monocytic cell line, THP-1 (145). In addition, Src is activated and associates with TLR3 upon stimulation with double stranded RNA in human monocyte derived DCs (154). Thus, the precise roles of Src family kinases in the various TLR signaling pathways leading to co-stimulatory molecule expression and cytokine production are complex and not yet fully understood. It is possible that the effect of CD45 on the MyD88 dependent pathway could be mediated via its dysregulation of Lyn, although the role of Lyn in TLR signaling remains poorly defined. In the $CD45^{-/-}$ BMDC, there was dysregulated phosphorylation of Lyn, Hck and Fyn implying that the effect of CD45 is not just restricted to Lyn.

4.3.3 Dysregulation of other molecules in $CD45^{-/-}$ TLR signaling

In addition to the SFK, the negative regulators Cbl and Dok2 showed aberrant phosphorylation, as did TLR3. Cbl has been shown to regulate the interaction of TLR4 with MyD88 (142) by mediating TLR4 ubiquitination and degradation. Classically, Cbl has also been shown to regulate the SFK through protein-protein interactions and by mediating their ubiquitination and degradation (155, 156). Phosphorylation of Cbl by SFK is important for its ability to act as an adaptor protein and to mediate its function as an E3 ubiquitin ligase and this interaction can result in the degradation of the SFK as well as of Cbl itself (157). It is possible that in the absence of CD45, due to lack of phosphorylation, Cbl is not fully able to potentiate degradation of the TLR-MyD88 complex and signaling through this pathway is prolonged. To further support the hypothesis that MyD88-dependent signaling is increased in

$CD45^{-/-}$ BMDC, examination of Cbl phosphorylation using a strictly MyD88-dependent agonist, such as Pam_3Csk_4, needs to be done. If Cbl is not activated upon TLR2 ligation in the $CD45^{-/-}$ cells then it would be possible to rule out any potential confounding contributions from the Trif pathway.

The LPS-dependent phosphorylation of Dok2 was completely abolished in $CD45^{-/-}$ BMDC. Dok was initially examined because it has been shown to complex with tyrosine phosphorylated SHIP1 (158). It was initially hypothesized that the constitutive hyperphosphorylation of SHIP would cause Dok recruitment and although this may be the case, the fact that Lyn is not activated upon LPS stimulation in the absence of CD45 may be the reason that Dok not being phosphorylated at all. Without phosphorylation, Dok cannot act as an adaptor and mediate downregulation. Interestingly, Dok is not activated in TLR2, 3 or 9 signaling (139) suggesting that neither the MyD88-dependent or independent pathways alone suffice to induce its activation. This restricts interpretation of the Dok finding to only the results obtained using LPS. Under these conditions a slight net decrease of cytokine secretion was observed. The hypothesis from these observations is that the loss of CD45 in the MyD88-independent pathway causes the loss of the IFNβ feedback loop that serves to augment cytokine secretion (133). To further test whether the dysregulation of Dok2 contributes to any enhancement in MyD88-dependent cytokine secretion, experiments would have to be done to remove the effects of Trif and IFNβ signaling. This could be accomplished by crossing the $CD45^{-/-}$ mice with the $Trif^{-/-}$ mice. (75).

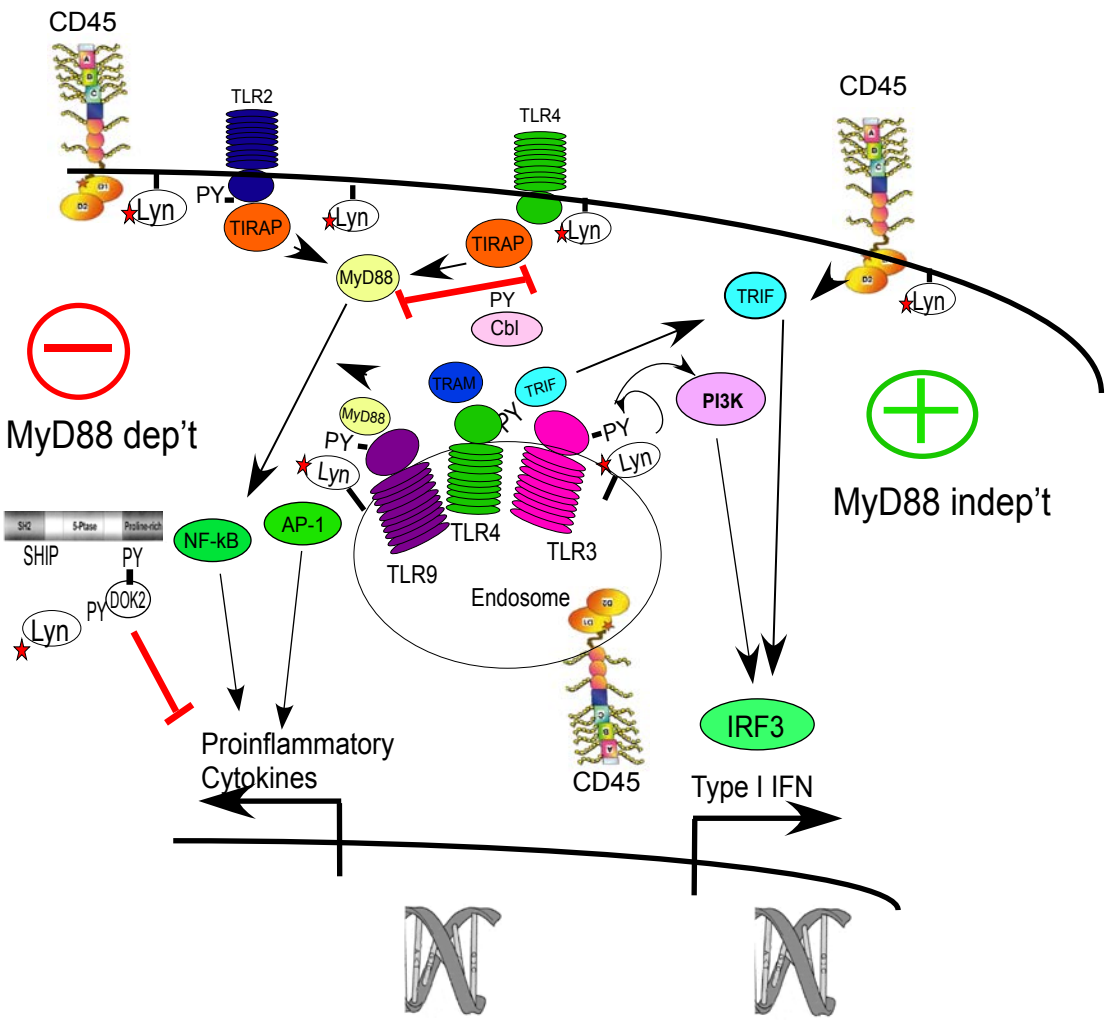
The last protein investigated and found to be hyperphosphorylated was TLR3. Experiments to immunoprecipitate TLR2 and TLR4 to determine their phosphorylation were inconclusive due in large measure to the lack of availability of quality antibodies that work

for immunoprecipitation coupled with low expression levels. A band the approximate molecular weight of TLR4 appeared hyperphosphorylated in the lanes corresponding to the CD45-/- BMDCs on the western blot but a suitable antibody to check for equal protein couldn't be found. As discussed in section 4.2.5, TLRs have several tyrosine residues and it is only in the last few years that their ability to be phosphorylated and their role in TLR signaling has been recognized. Hyperphosphorylation of the TLR would be predicted to enhance the end result of signaling which, in the case of TLR3, would be IFNβ production. Clearly this is not the case, however, it is possible that phosphorylation of some residues could act as scaffolds to assemble negative regulators or that lack of phosphorylation on the residues necessary to mediate IRF3 activation is obscured by hyperphosphorylation on others. To date, the phosphatase responsible for removing the phosphorylation has not been identified and the fact that the TLR is hyperphosphorylated in the absence of CD45 raises the intriguing possibility that the TLRs might function as a substrate for CD45 in innate cells just as CD3 has been implicated as a substrate in T cells (159).

4.3.4 Role for CD45 in TLR signaling

In this study, CD45 acting through its substrates such as Lyn, has been implicated in TLR signaling in DCs. Although some answers have been obtained, many more intriguing questions have been raised. Although knowledge of TLR signaling has advanced rapidly since the first discovery of MyD88 as an essential adaptor in TLR signaling, there is still much that remains unknown including how and where other pathways, such as integrin signaling, intersect and influence TLR signaling (67, 160). Although work still remains to be

done to fully characterize the function CD45 has within TLR signaling, this study has been able to discern that in the absence of CD45, Lyn is not activated upon LPS stimulation and that this may in turn alter the function of Lyn substrates such as Cbl and Dok that are involved in the negative regulation of MyD88 signaling as outlined in figure 4.9. The hypothesis is still that CD45 is a negative regulator of MyD88 dependent signaling and a positive regulator of the Trif pathway although more definitive studies in the future, looking more closely at IRF3 activation and TLR phosphorylation would help to support this idea. Due to lack of good reagents for murine cells, our experiments to determine IRF3 phosphorylation were not successful.



4.9 Schematic rendering of the role of CD45 and Lyn in TLR signaling.

Chapter 5: A Role for CD45 in innate cell downstream functions

5.1 Introduction

Having previously identified a role for CD45 in TLR-driven cytokine production from dendritic cells, the next step was to determine if there would be a functional consequence. Dendritic cells have the important job of initiating the adaptive immune response and they also play a vital part in directing the outcome of the T cell response through expression of costimulatory molecules and their cytokine secretion patterns. Cytokines like IL-12 play an important role mediating IFNγ release from T cells and directing CD4 T cells towards a Th1 response. However, T cells aren't the only cell type that DCs interface with. NK cells are members of the innate immune system that eliminate infected and altered cells in the body. Cross-talk with activated DCs permits NK cell expansion, cytolytic function and IFNγ secretion. Just like for T cells, IL-12 secreted by activated DCs is a main factor in governing IFNγ secretion by NK cells (20). In this chapter, the ability of TLR-activated $CD45^{-/-}$ BMDC to drive IFNγ production from T cells and NK cells was tested.

Cytokines are not only important for DCs. When proinflammatory cytokines are secreted in excessive amounts they cause severe conditions like septic shock. The main mediator of septic shock is TNFα, the systemic secretion of which can result from bacteria entering the blood stream and being distributed all over the body. Components of the bacteria stimulate the production of TNFα when recognized by the TLR. Septic shock is a leading cause of death in hospital intensive care units with a 50% mortality rate. Since the lack of

CD45 alters TNFα secretion upon TLR ligation, this chapter also sought to determine if the absence of CD45 would change the outcome in a mouse model of sepsis.

5.2 Results

5.2.1 LPS stimulated CD45$^{-/-}$ BMDC are less at efficient stimulating NK cells to produce IFNγ

Dendritic cell derived IL-12p70 has also been shown to influence NK cell activation and subsequent IFNγ secretion (20). Therefore the ability of the CD45$^{-/-}$ DCs to induce IFNγ from NK cells was investigated. Figure 5.1 shows that NK cells co-cultured with Ultrapure LPS activated CD45$^{-/-}$ BMDC produced less IFNγ than those cultured with LPS activated CD45$^{+/+}$ DCs. However, this was only seen when dendritic cells were activated with 1 μg/ml LPS, and not at 100ng/ml of LPS where a significant decrease in IL-12 production was observed in CD45$^{-/-}$ DCs (data not shown). This raises the possibility that other factors other than IL-12 may contribute to this effect. No IFNγ was detected when NK cells were incubated with unstimulated BMDC or with LPS activated BMDC alone or NK cells alone plus or minus LPS (data not shown). These data indicate that LPS stimulated CD45$^{-/-}$ dendritic cells have a reduced ability to activate NK cells to secrete IFNγ at 1μg/ml of LPS.

5.2.2 LPS stimulated CD45$^{-/-}$ BMDC are less efficient at priming IFNγ from CD4+ T cells

Since DC derived IL-12 is instrumental in directing the adaptive immune response towards a Th1 response, T cells were activated *in vitro* with CD45$^{+/+}$ and CD45$^{-/-}$ BMDC to determine if the loss of CD45 affected the generation of a Th1 response and subsequent IFNγ

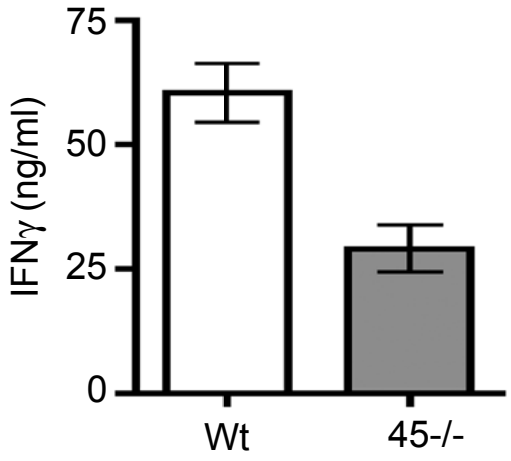


Fig 5.1 Ability of CD45$^{-/-}$ BMDC to induce IFNγ from NK cells. IFNγ release from NK cells cultured *in vitro* with BMDC. BMDC and NK cells were co-cultured in the presence or absence of 1 μg/ml LPS for 18 hours and IFNγ in the culture supernatant was measured by ELISA. The mean ± SEM from a representative experiment using three mice per condition is shown, ** $p < 0.01$. Four independent experiments using a total 14 mice were performed.

production. CD4 T cells isolated from TCR transgenic mice specific for $OVA_{323-339}$ peptide (OTII) were incubated with peptide loaded, LPS (100 ng/ml Ultrapure) activated BMDC and co-cultured for 6 days. The cells were then re-stimulated for 3 hours with PMA/ionomycin and the production of IFNγ measured by intracellular staining in $CD4^+$ cells. Stimulation with LPS activated $CD45^{-/-}$ DCs generated a lower percentage of $CD4^+$ T cells producing IFNγ over a range of peptide concentrations (fig 5.2a). The average fold increase in $CD4^+$ T cells making IFNγ was consistently less when LPS activated $CD45^{+/+}$ and $CD45^{-/-}$ dendritic cells were compared (fig 5.2b). This decrease in IFNγ production was not accompanied by an increase in IL4 production, as no intracellular IL4 was detected by flow cytometry (data not shown). This reduced activation of Th1 cells by LPS stimulated $CD45^{-/-}$ DCs, as measured by IFNγ production, indicates a functional defect in LPS activated $CD45^{-/-}$ DCs and is consistent with the LPS stimulated $CD45^{-/-}$ DCs producing less IL-12p70 at this LPS concentration.

5.2.3 Pam_3Csk_4 stimulated $CD45^{-/-}$ BMDC are more efficient at stimulating T cells to secrete IFNγ

IL-12 production by DCs is known to provide a favorable environment for the development of Th1 cells that produce IFNγ upon activation. In figure 5.2 it was demonstrated that LPS stimulated $CD45^{-/-}$ BMDC were less efficient at inducing IFNγ secretion from co-cultured OTII T cells following re-stimulation. This was consistent with the reduced amount of IL-12 produced. To determine if Pam_3Csk_4 and CpG stimulated $CD45^{-/-}$ BMDC, which produce significantly more IL-12 than wild-type BMDC, were more

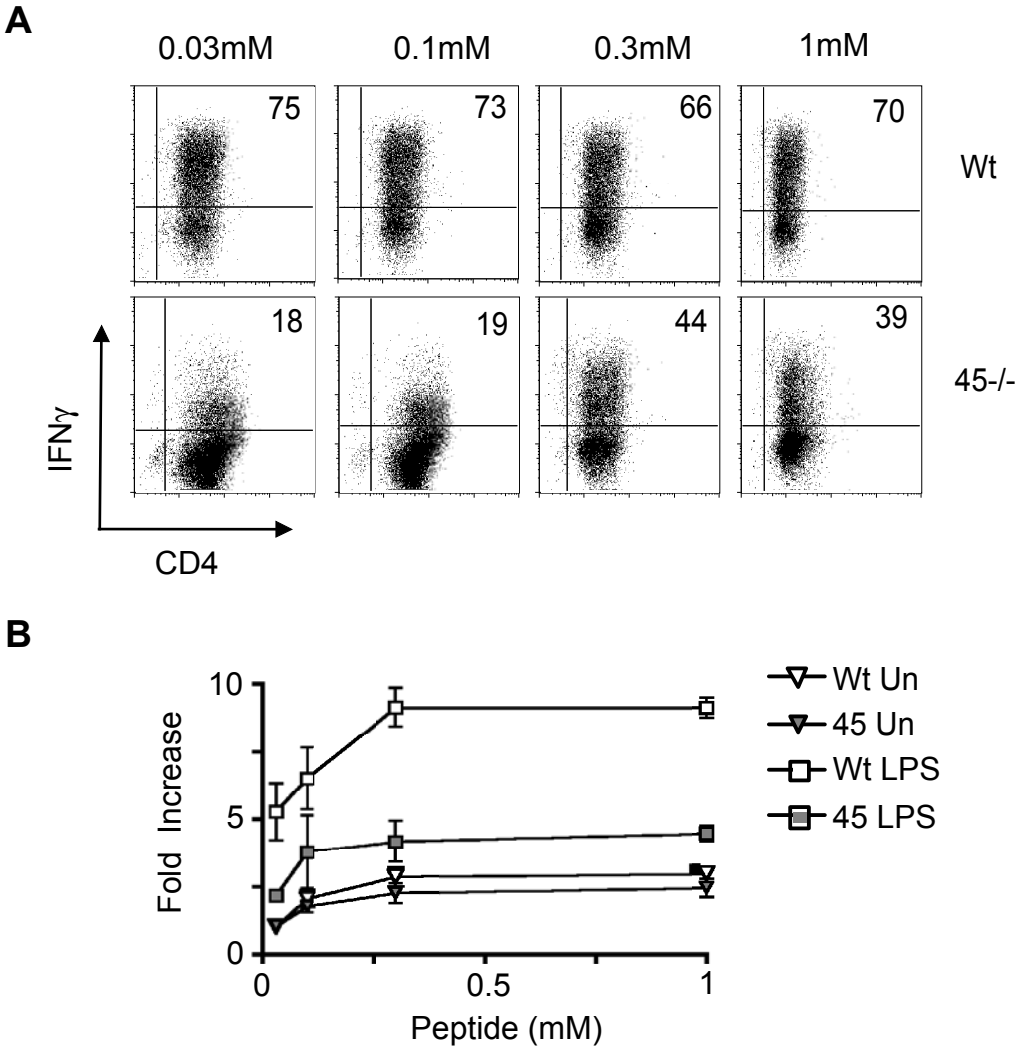


Fig 5.2 Ability of CD45$^{+/+}$ (Wt) and CD45$^{-/-}$ dendritic cells to stimulate IFNγ production from T cells. (A) Flow cytometric analysis of IFNγ secretion from T cells co-cultured with LPS activated BMDC. OTII CD4 T cells were co-cultured with purified unstimulated or LPS-stimulated BMDC for 6 days prior to re-stimulation with PMA/ionomycin to measure intracellular IFNγ. Experiment is one representative of 3. (B) Graphical representation of the mean fold-increase (± SEM) in IFNγ secretion by OTII T cells normalized to the amount of IFNγ produced in OTII T cells incubated with unstimulated CD45$^{+/+}$ dendritic cells pulsed with 0.03 μM peptide.

effective than wild-type DCs at generating IFNγ producing Th1 cells, stimulated dendritic cells were co-cultured with OTII T cells for three days and IFNγ production measured by ELISA. Figure 5.3a demonstrates that after 3 days in co-culture, OTII cells cultured with LPS (100 ng/ml Ultrapure) activated $CD45^{-/-}$ BMDC produce less IFNγ whereas Pam_3Csk_4 activated $CD45^{-/-}$ BMDC induced higher levels of IFNγ (fig 5.3b). This is consistent with the levels of IL-12 they produce. IL-2 levels from the LPS activated $CD45^{-/-}$ BMDC: T cell coculture were similar, if not slightly increased at the lower peptide concentrations, suggesting that the defect in IFNγ production is not due to an inability to drive T cell proliferation (fig 5.3a). IL-2 secretion in the $CD45^{-/-}$ Pam_3Csk_4 BMDC coculture was actually slightly decreased indicating that T cell activation and proliferation was not as efficient. This makes the increase in IFNγ even more significant.

Surprisingly, OTII T cells cultured with CpG activated $CD45^{-/-}$ BMDC produced similar amounts of IFNγ to wild-type BMDC, which was not consistent with their increased production of IL-12 (fig 5.3c). However, this may be related to the increased amount of IFNβ produced by CpG stimulated $CD45^{-/-}$ BMDC (approximately 3 fold more than wild-type CpG stimulated BMDC) as IL-2 levels were significantly reduced in the co-cultures with $CD45^{-/-}$ BMDC (consistent with the ability of IFNβ to inhibit T cell proliferation (161)). This demonstrates the ability of $CD45^{-/-}$ BMDC to affect the amount of cytokine secreted by activated T cells and suggests a role for CD45 in modulating Th1 responses, possibly via its ability to modulate TLR induced secretion of pro-inflammatory cytokines in DCs.

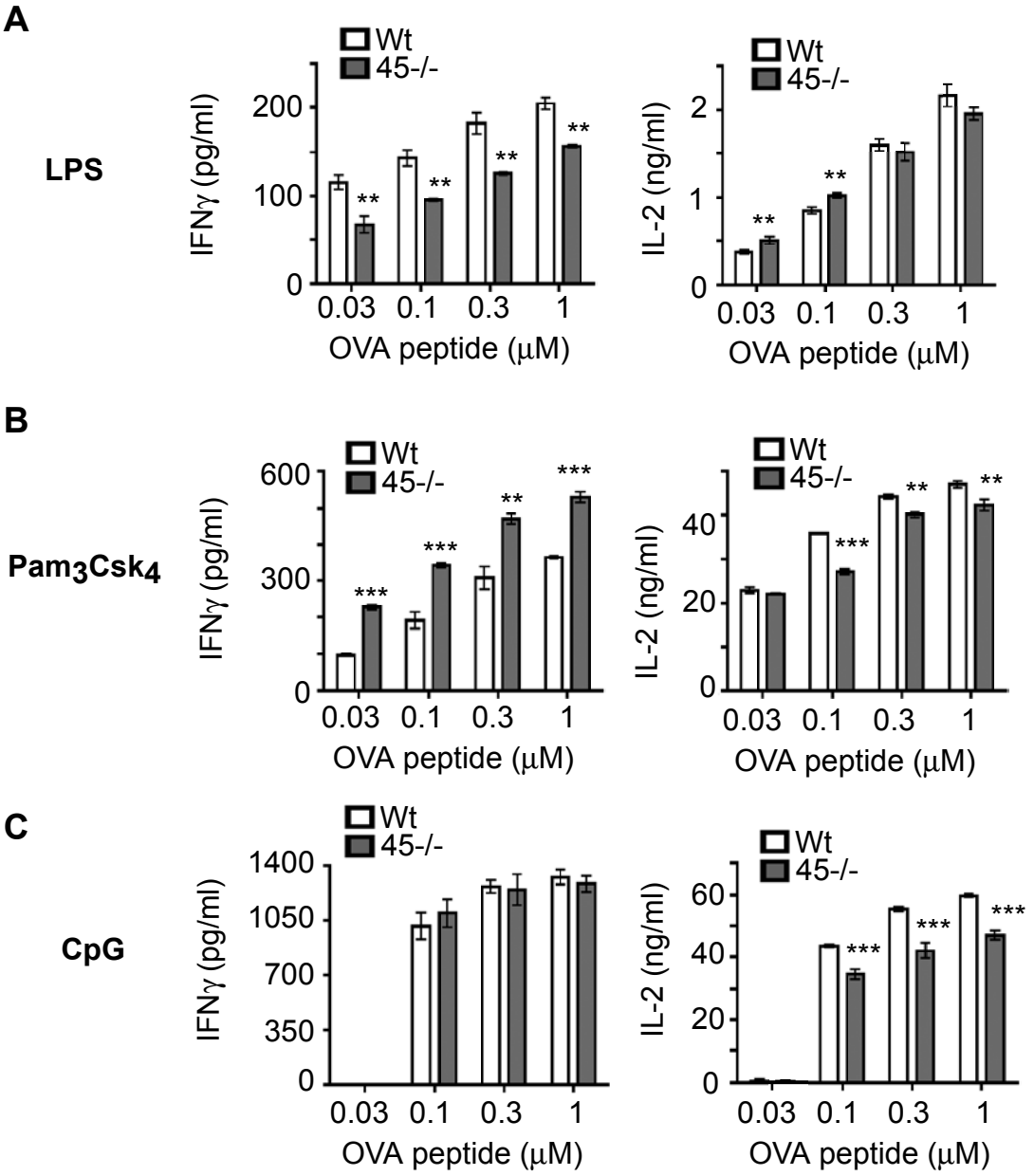


Fig 5.3 IFNγ and IL2 secretion from cocultures of OTII T cells and LPS, CpG or Pam_3Csk_4 activated BMDC. Purified day 8 BMDC were activated with (A) 100ng/ml Ultrapure LPS, (B) 1μM CpG or (C) 250ng/ml Pam_3Csk_4 in the presence of titrated doses of OVA peptide prior to the addition of CD4+ OTII T cells. After 72 hours in co-culture, supernatants were harvested for analysis by IFNγ ELISA and IL2 ELISA. Experiments are representative of 3 repeats using BMDC pooled from 3 mice, ** $p \leq 0.001$, *** $p \leq 0.0001$.

5.2.4 CD45$^{-/-}$ mice have increased serum TNFα and decreased resistance to high doses of LPS

A mouse model for sepsis involves the injection of high doses of LPS to mimic the effects of a systemic infection. Since CD45$^{-/-}$ BMDC secreted less TNFα upon LPS stimulation, it was of interest to see if this finding had a broader *in vivo* implication and if the CD45$^{-/-}$ mice would be more resistant to the effects of high levels of endotoxin. Mice were injected with 10mg/kg UltraPure LPS to induce sepsis and the condition of the mice was monitored over a period of 72 hours. The dose was intended to be lethal however, even at 20mg/kg, no mice exhibited signs of morbidity so symptoms were scored for severity according to the UBC Rodent Monitoring System. Surprisingly, the CD45$^{-/-}$ mice had more severe symptoms than their C57BL/6 counterparts and they did not dissipate as quickly (fig 5.4a). Since the hypothesis was that the CD45$^{-/-}$ mice would be more resistant to septic shock based on decreased TLR4-driven cytokine secretion, serum TNFα was assessed at one and three hours post-injection. Consistent with the severity of septic shock symptoms, the CD45$^{-/-}$ mice had a significant spike in serum TNFα at 1 hour post-injection (5.4b) but by three hours both wild type and knockout mice had a similar concentration of TNFα and both were returning to baseline levels.

5.2.5 CD45$^{-/-}$ mice have decreased survival in a low-dose sepsis model

To determine if the increase in TNFα in the CD45$^{-/-}$ mice at one hour post-injection was biologically significant, the hepatotoxin D-galactosamine was co-injected with a low dose of LPS (162). D-galactosamine sensitizes the cells of the liver to TNFα released upon

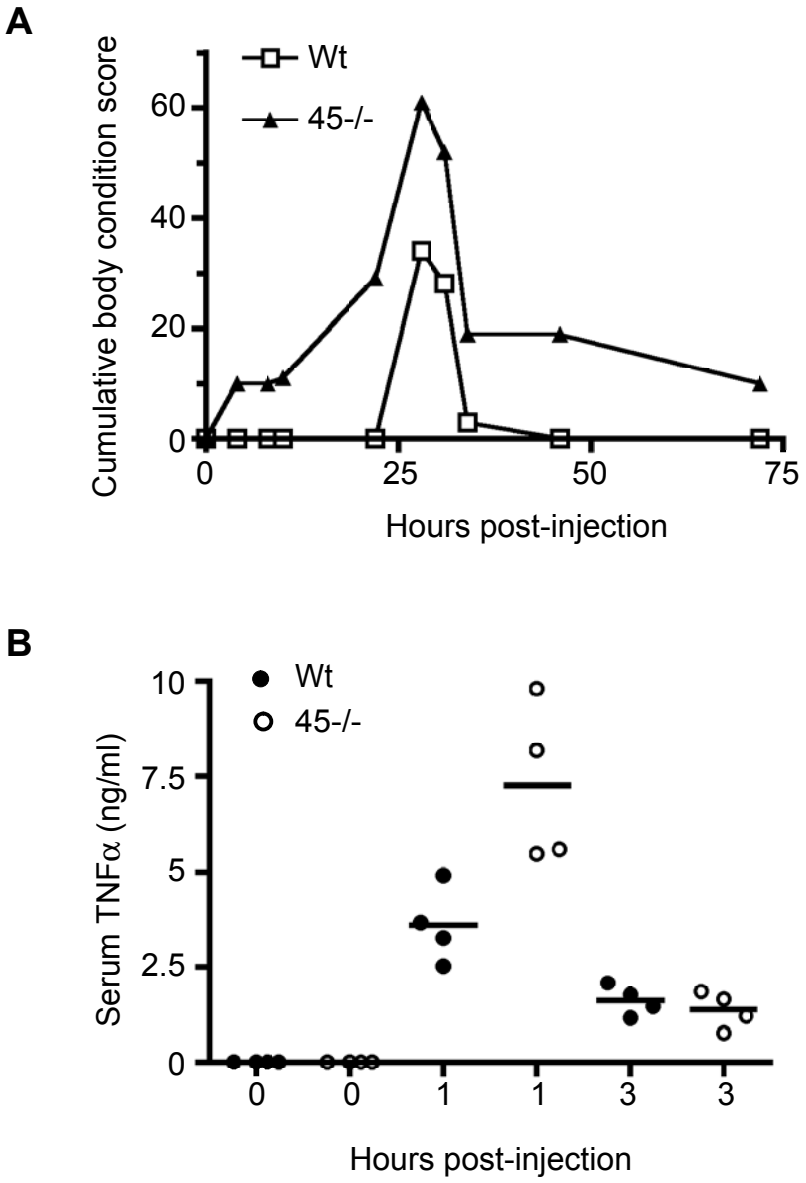


Fig 5.4 CD45$^{-/-}$ mice have more severe symptoms of LPS-induced toxic shock. (A) Five each of C57BL/6 (Wt) and CD45$^{-/-}$ female mice were injected with 10mg/kg of UltraPure LPS and monitored at intervals for three days. Cumulative scores were determined based on the severity of symptoms observed in the mice at each time point according to criteria set out by in the Rodent Scoring system. (B) Serum was obtained by cardiac puncture from mice injected with 10mg/kg of UltraPure LPS at the timepoints indicated. Serum TNFα was analyzed by ELISA and the results from each mouse are shown on the graph as a single dot. At 1 hour post-injection the CD45-/- mice have significantly higher (p=0.008) TNFα. The experiment is one representative of 3.

LPS injection and the mice succumb rapidly to liver failure. As shown in figure 5.5, CD45-/- mice had a significantly higher rate of mortality with all of the mice having to be euthanized by 7 hours post-injection. In contrast, the C57BL/6 group still had 25% of the mice still alive by 9 hours post-injection when the experiment was terminated. This result shows that the increased serum TNFα in the $CD45^{-/-}$ mice is significant enough to have biological consequences in this model.

5.2.6 $CD45^{-/-}$ mice have more peritoneal macrophages

The result obtained with the *in vivo* LPS injections was contrary to the observed decrease in TNFα in DCs. Therefore it was of interest to try to determine the reason for the increase in the *in vivo* TNFα in the $CD45^{-/-}$ mice. In the above experiments, LPS was injected intraperitoneally into the mice so it was logical to start by analysis of the cells present in the peritoneal cavity of the CD45 knockout mice. Uninjected mice were sacrificed and the cells collected by peritoneal lavage, counted and analyzed by flow cytometry. The $CD45^{-/-}$ mice had approximately 2-fold more cells present in the cavity (fig 5.6a). In septic shock, macrophages play a large role in the secretion of the TNFα and they make up a large percentage of the cells found in the peritoneal space. By flow cytometry, the frequency of macrophages was similar in the C57BL/6 and in the $CD45^{-/-}$ mice (fig 5.6b). However, when the total number was calculated there were nearly 2-fold more macrophages present (fig 5.6c). The fact that the macrophage population is double in the knockout mice is a likely reason for the increased serum TNFα but further experiments would be necessary to

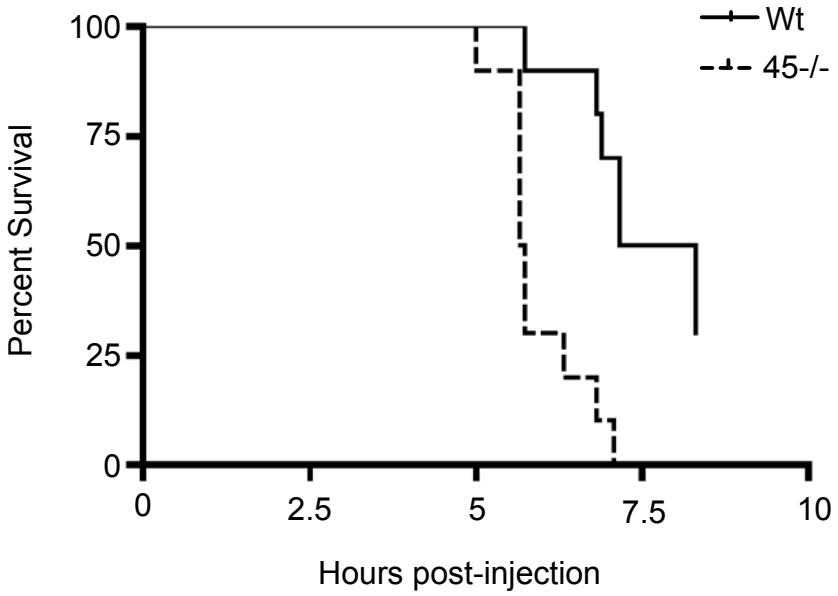


Fig 5.5 CD45$^{-/-}$ mice have increased susceptibility in a low-dose model of LPS-induced sepsis. Groups of 10 C57BL/6 (Wt) and CD45$^{-/-}$ mice were injected with 20ug of UltraPure LPS in conjunction with 20mg of d-galactosamine. Mice were monitored hourly for signs of morbidity such as non-responsiveness or immobility at which point they were euthanized and the time logged. CD45$^{-/-}$ mice had a significantly ($p=0.0002$ by log-rank test) higher rate of death than their Wt counterparts. The experiment was terminated at 9 hours post-injection. This experiment is representative of 3 independent repeats with that used at least 5 mice each.

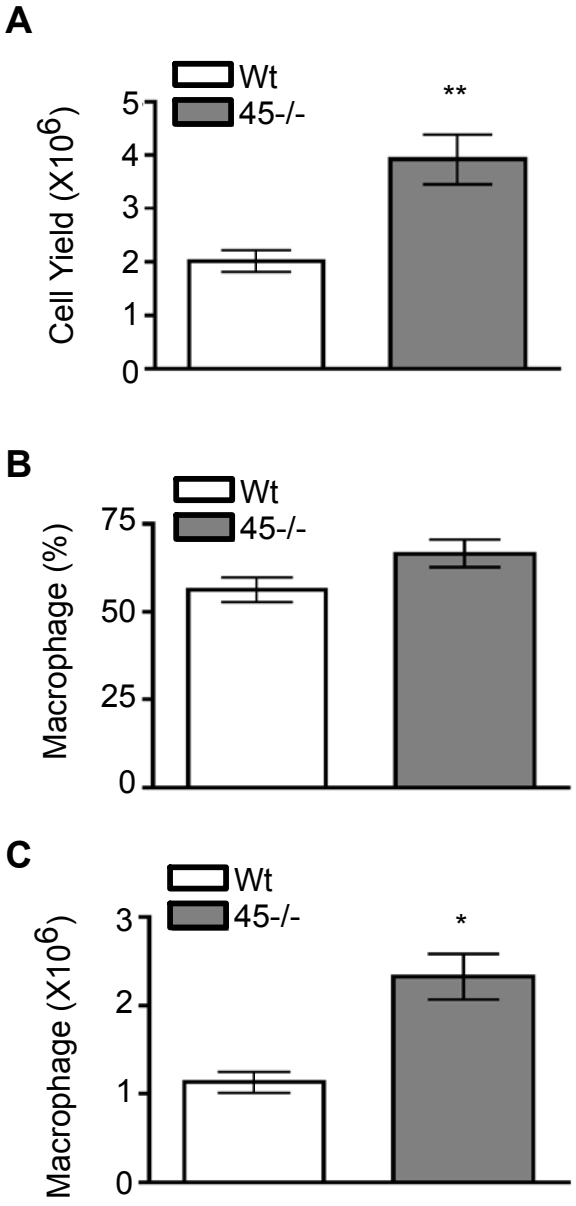


Fig 5.6 Increased macrophage numbers in the CD45$^{-/-}$ peritoneal cavity. The peritoneal cavities of C57BL/6 (Wt) and CD45$^{-/-}$ mice (45-/-) were flushed with 5ml of wash solution to recover the cells present. The red blood cells in the suspension were lysed and the remaining cells were counted by hemocytometry and prepared for flow cytometric analysis (FACS). (A) Total cells recovered from Wt and 45-/- mice (p=0.001). (B) Frequency of macrophages by FACS. (C) Total number of macrophages calculated by multiplying the macrophage frequency by the total cell number (p=0.004).

determine if these macrophages are in fact making less TNFα per cell as the DC data indicates.

5.3 Discussion

5.3.1 Data summary

The previous chapters outlined a role for CD45 in TLR signaling in DCs and hinted at mechanistic possibilities. The goal of this chapter was to determine, as much as possible, if these results would have meaning in shaping the immune response and if they have any *in vivo* relevance. The hypothesis was that alterations in IL-12 secretion in the $CD45^{-/-}$ BMDCs would result in changes in IFNγ secretion from T cells and NK cells. LPS activated $CD45^{-/-}$ BMDCs cultured with NK cells or T cells do result in less IFNγ made although they do seem comparable in their ability to activate the T cells, evidenced by comparable amounts of IL-2 secreted into the culture media. Pam_3Csk_4 activated, CD45-deficient BMDCs produced more IL-12 and induced higher IFNγ from the T cells even in the face of a decreased ability to drive their expansion. Results from the CpG activated BMDCs were less clear as there was substantially less IL-2 produced in the cocultures with the $CD45^{-/-}$ BMDCs and similar amounts of IFNγ. It is possible to extrapolate and say that if T cell activation was the same, the $CD45^{-/-}$ BMDCs would likely be driving a much higher level of IFNγ production.

To broaden the focus a little and to determine some *in vivo* relevance for CD45 in TLR signaling, *in vivo* models of LPS-driven sepsis were examined. $CD45^{-/-}$ mice unexpectedly had more severe symptoms and higher serum TNFα than the wild type mice. The increase in serum TNFα was determined to be biologically significant in a low-dose model of septic shock with the $CD45^{-/-}$ mice having a significantly reduced survival rate. Examination of the composition of the peritoneal cells revealed a two-fold increase in the

total cellularity and in the number of macrophages found in the $CD45^{-/-}$ mice. Without further experiments it is difficult to know for sure but it is possible that the large difference in macrophage numbers may play a large role in the observed increase in TNFα.

5.3.2 CD45 in NK and T cell activation

The hypothesis that the lack of CD45 in DCs alters TLR-mediated proinflammatory cytokine secretion leading to alterations in the ability of those DCs to initiate an appropriate immune response was supported by experimental data obtained in this chapter. NK cell cocultures with TLR4-activated BMDC yielded less IFNγ when CD45 was not present on the DCs. Interestingly this occurred not at the predicted 100ng/ml concentration but at 1μg/ml. This raises the point that factors beyond IL-12 also contribute to NK activation by DCs and in recent studies cytokines such as IL-15 (163) and IL-2 (19) have come to the fore. Adding to the complexity, presentation of IL-15 to NK cells by DCs is a process augmented by the secretion of type I IFNs (164) which is also reduced in TLR4-activated $CD45^{-/-}$ BMDCs. It would be interesting to see what effect loss of CD45 has on both IL-15 and IL-2 in BMDCs at different doses of LPS in order to determine if alterations in these factors may also be contributing to the decrease in NK cell IFNγ in the $CD45^{-/-}$ cocultures. Since NK cells are involved in eliminating aberrant autologous cells indicative of cancerous states or of viral infection and early IFNγ secretion by NK cells is important for early antiviral defense (165), in a mouse model where DCs were deficient in CD45 one might expect impaired ability to control the virus in the early stages of infection resulting in higher mortality.

The ability of $CD45^{-/-}$ DCs to influence T cell activation in a stimulus-dependent manner was unexpected. Costimulatory molecule assessment suggested that, if anything, $CD45^{-/-}$ BMDC would be more potent T cell activators however, in the case of TLR2 and TLR9 activated knockout BMDCs, IL-2 secretion was decreased. CpG induced very high levels of IFNβ from $CD45^{-/-}$ BMDCs and, as discussed in section 5.2.2, type I IFNs have documented antiproliferative and proapoptotic functions (166) in many cell types, including T cells. In contrast, type I IFNs are also important for maintaining the survival and expansion of activated CD4+ and CD8+ T cells (167). The difference seems to lie with the activation state of the T cell at the time of exposure to the type I IFN (168), resting T cells are subject to antiproliferative activities, whereas activated cells can downregulate responses to type I IFNs and can expand and proliferate in its presence. It is possible that the decreased IL-2 in the CpG activated $CD45^{-/-}$ BMDC: T cell cultures is an artifact caused by buildup of type I IFN in the coculture plate and the time of addition of the stimulated DCs to the T cells. *In vivo* experiments would be beneficial to validate T cell activation by CpG activated BMDCs deficient in CD45.

In the case of the Pam_3Csk_4 cocultures, the very slight decrease in IL-2 observed at lower peptide concentrations cannot be attributed to type I IFN production because none was produced upon TLR2 ligation (data not shown). The decreased IL-2 does suggest that the Pam_3Csk_4 activated $CD45^{-/-}$ BMDC are suboptimal for stimulating T cell activation when peptide is limiting. Since the concentration of Pam_3Csk_4 was consistent for all the peptide concentrations, the effect on T cell activation may instead be caused by inefficiencies in stimulation as opposed to cytokine secretion. Costimulation lowers the activation threshold for T cells and allows them to be more responsive at lower antigen concentrations. No

significant differences were observed in the upregulation of either CD80 or CD86 in TLR2 activated $CD45^{-/-}$ BMDCs but it is possible that upregulation of a different costimulatory molecule (for example ICOSL, OX-40L or CD70) could be altered, making activation of T cells less efficient at suboptimal peptide concentrations.

5.3.3 In vivo sepsis models in $CD45^{-/-}$ mice

To evoke a broader implication for decreased LPS-driven proinflammatory cytokine secretion in $CD45^{-/-}$ cells, the role of CD45 in septic shock was examined. Contrary to expectations, the mice devoid of CD45 produced more TNFα and were more susceptible to symptoms of LPS-induced septic shock and their recovery was slower. Plasma TNFα is correlated directly with the severity of the shock (169) and resulting multi-organ failure. $CD45^{-/-}$ mice showed a biologically significant elevation in serum TNFα correlating with the increased severity in symptoms in these mice. This was a very interesting finding until it was determined that the $CD45^{-/-}$ mice actually had higher numbers of macrophages in their peritoneal cavities. Since T cells make up a very tiny percentage of the cells in the peritoneal cavity, it is unlikely that the increase in macrophages is due to an expansion of the population into the void left by the lack of T cells.

One possibility for increased numbers of macrophages is that there is increased traffic to the cavity. CD45 and Src-family kinases have identified roles in integrin signaling. CD11b is a β2-integrin subunit expressed at high levels on myeloid cells like neutrophils and monocytes and plays an important role in their ability to extravasate into tissues (170) through interactions with intracellular adhesion molecule-1 (ICAM-1) and -2. In a yeast two-

hybrid screen, CD45 was found to interact with a related integrin, CD11a (171) so it is plausible that through regulation of integrin-mediated adhesion, monocytes are either better able to extravasate from circulation into the peritoneum or perhaps are retained there. Alternatively, it is also a possibility that the absence of CD45 leads to an increase in the numbers of macrophages. Work done in this thesis has suggested that Lyn is not active in the absence of CD45 and, in a phenotype somewhat similar to the $CD45^{-/-}$ mice, $Lyn^{-/-}$ also develop splenomegaly and have enhanced numbers of macrophages. There may even be a combined effect of these two scenarios that cause the 2-fold increase in peritoneal macrophage numbers in the peritoneal cavity of $CD45^{-/-}$ mice.

Chapter 6: Summary and future work

6.1 Summarizing the role for CD45 in innate immunity

This work has identified a role for CD45 in DCs. Specifically; DCs lacking CD45 show altered proinflammatory cytokine secretion patterns that vary with the TLR ligated. The working model is that CD45 is a negative regulator of MyD88-dependent signaling and a positive regulator of the Trif pathway. The model can be summarized as follows. CD45 acts on Src-family kinases, such as Lyn, that participate in signaling at the level independent of, and upstream from, the TLR (145). Loss of CD45 leads to inactivation of Lyn that regulates other regulators of TLR signaling like Cbl (142, 172) and potentially even other pathways that feed into the TLR signaling and influence downstream proinflammatory cytokine production such as integrins (67, 173, 174). Alterations in TLR-activated CD45-/- BMDCs result in inappropriate induction of IFNγ from NK cells and changes in T cell activation and IFNγ production. *In vivo*, injection of LPS actually leads to increased serum TNFα and an increased severity in septic shock symptoms. This outcome is likely a result of the increased number of macrophages present in the CD45 knockout mice. The schematic model of where CD45 fits into the innate immune response is summarized in figure 6.1.

6.2 A role for CD45 in DC development and activation

Unlike T cells and B cells, the absence of CD45 does not impair the number of DCs that accumulate in the CD45 knockout mice. This finding is not wholly unexpected, as DCs

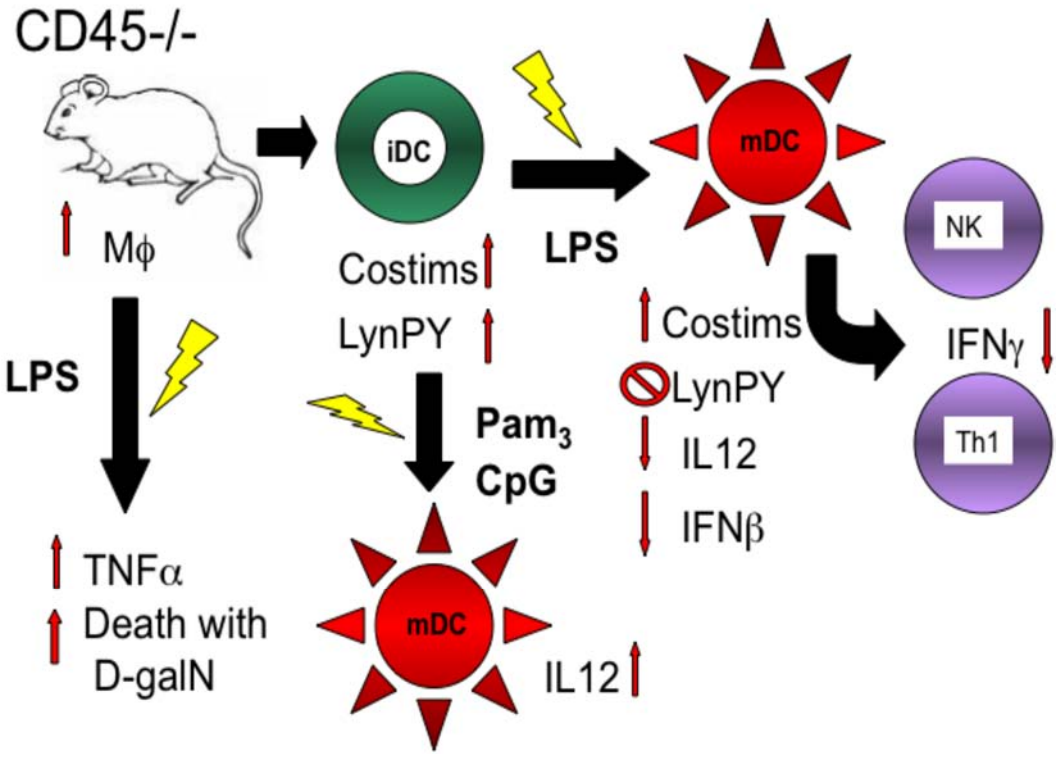


Fig 6.1 Diagram summarizing the role of CD45 in TLR-stimulated responses and its influence on the adaptive response.

do not rely on antigen receptor signaling for their development. What was more interesting was the inversion in the frequencies of the $CD8\alpha^+$ and $CD11b^+$ populations in the absence of CD45. Initially these two populations were designated "lymphoid" and "myeloid" to denote their hematopoietic origins. This is now known to be false as both populations can arise from both the common lymphoid and the common myeloid progenitor (175). However, there seem to be distinct requirements for different transcription factors that regulate lineage decisions in DC suggesting that they do represent two distinct populations (10). The interferon-regulatory factors (IRFs) play a major role in DC development and lineage commitment (176) with IRF8 being vital for the development of $CD8\alpha^+$ DCs (176, 177). Interestingly, IRF8 also drives macrophage differentiation at the expense of granulocyte development (178, 179) and relies upon tyrosine phosphorylation by Jak2 for activation (180). Analysis of the composition of the spleen of the $CD45^{-/-}$ mice showed an increase in the number of macrophages and a decrease in the number of granulocytes in addition to the increase in $CD8\alpha^+$ DCs. It is possible that CD45 regulates IRF8 activation through its influence on Jaks leading to alterations in hematopoietic lineage decisions. Experiments to address this possibility could yield novel information about both IRF8 activation and the role of CD45 in the *in vivo* development of myeloid cells.

In the controlled conditions *in vitro*, CD45 did not play a dramatic role in the development of GM-CSF derived BMDCs. Even though published research by Roach *et al*., (111) suggested that lack of CD45 altered the adherence properties of BMDMs on plastic culture dishes, this did not seem to be a factor in BMDC development. In figure 3.7 there is a slight increase in the number of $CD11c^{hi}$ cells found in the adherent fraction of the cultures at earlier days but this was not observed after day 6 whereas $CD45^{-/-}$ bone marrow-derived

macrophages (BMDMs) seem to have a defect in long-term adherence to the plastic resulting in more non-adherent cells (111). β2-integrin signaling through plastic adhesion does not seem to play the same role in BMDC development as it does in BMDMs.

$CD45^{-/-}$ BMDCs and splenic DCs that had not been exposed to activating signals showed slightly elevated levels of costimulatory molecules suggesting that CD45 may play a minor role in governing the partial maturation process. This data is substantiated by Piercy *et al*., as they observed similar elevated levels of CD40 and CD86 in their unstimulated $CD45^{-/-}$ BMDC (120). Data have suggested that partial maturation of DCs is influenced by E-cadherin (181). DCs in culture cluster with each other via E-cadherin interactions and *in vivo* it is likely that DC in peripheral tissues interact with neighboring cells by the same mechanism. Disruption of E-cadherin interactions led to partial upregulation of costimulatory molecules like CD86 on the cell surface. Src-family kinases have been shown to play a role in cadherin signaling (182). In cells where SFK activity was decreased by overexpression of Csk, E-cadherin cell-cell adhesion increased. In $CD45^{-/-}$ DCs if SFK activity is dysregulated, decreased cell-cell adhesion could occur, leading to partial maturation.

The most important finding of this thesis is that the absence of CD45 had a differential effect on the patterns of TLR-driven cytokine secretion. The MyD88-dependent pathways of TLR2 and TLR9 showed enhanced cytokine secretion while the MyD88-independent TLR3 pathway had decreased IFNβ production. This led to the hypothesis that CD45 is a negative regulator of MyD88-dependent signaling and a positive regulator of the Trif pathway. The TLR4 pathways showed a slight concentration-dependent effect; at 10ng/ml there was a small but significant increase in cytokine and at 100ng/ml there was a slight but significant decrease.

TLR4 is the most complex of the TLR signaling pathways and uses both MyD88 and Trif to signal to produce proinflammatory cytokines and IFNβ, respectively. These two pathways do not exist in cellular voids; there is evidence in the literature of cross talk between the two such that IFNβ production serves to augment proinflammatory cytokine secretion (133) as well as its own production. It is possible that in the absence of CD45, signaling to produce IFNβ is suboptimal and thus its ability to augment proinflammatory cytokine production is diminished. On the other hand, the absence of CD45 causes an increase in the pathway leading to proinflammatory cytokine production. At 100ng/ml of LPS, where both IFNβ and cytokines are produced, the net result is only a slight decrease in cytokine as the increased MyD88 signaling is able to compensate for a decreased IFNβ-dependent feedback loop. At 10ng/ml, where there is no detectable IFNβ and thus no effect of the Trif pathway on cytokine production, the net observable result is the contribution of only the MyD88 dependent pathway and there is a visible increase in proinflammatory cytokines.

Further experiments would help to support or disprove this hypothesis. An attempt was made to block IFNβ signaling through the type I IFN receptor by adding IFNβ neutralizing antibodies into the culture wells. However, use of the isotype control antibody alone (even without LPS) caused activation of the BMDC and resulted in cytokine secretion. DCs can be induced to mature and produce IL-12 through ligation of their FcRs (183) but it is also a possibility that the antibody solution contained some endotoxin. Without differentiating between the two scenarios, interpretation of the data is difficult. A future possibility to determine if a decrease in the Trif pathway is cancelling out an increase in the MyD88-dependent pathway would be to cross the $CD45^{-/-}$ mice with the Trif knockout mice.

It might also be of interest to cross the CD45$^{-/-}$ mice with the type I interferon receptor (IFNAR$^{-/-}$) mice (184) to help determine if signaling through Trif is impaired or if the decrease in IFNβ is a result of inadequate signaling through IFNAR to amplify IFNβ production. Since CD45 has been implicated as a regulator of Jaks and cytokine signaling (81), the possibility exists that type I IFN signaling is disrupted. However, in the work done by Irie-Sasaki *et al.*,(81) Jaks were found to be hyperphosphorylated in the absence of CD45 and cytokine signaling was actually enhanced. A contrasting paper by Petricoin *et al*., found that Jaks were not affected by the absence of CD45 (185). As shown in figure 4.1, neither Jak1 nor Jak2 was hyperphosphorylated in BMDC. If CD45 were in fact a negative regulator of IFNβ signaling in BMDC, increased IFNβ would result and instead the converse was observed.

6.3 CD45 and TLR signaling

Analysis of TLR signaling was conducted in order to find a mechanism for the differential cytokine secretion observed in the CD45$^{-/-}$ DCs. It is likely that at least a good portion of the effects observed in CD45$^{-/-}$ DCs are a result of dysregulation of the SFKs. To date no studies have been able to place the SFKs squarely in the TLR pathway although it is accepted in the literature that they do play a role in TLR signaling. Although there were perturbations in the phosphorylation state of at least two proteins in unstimulated DCs lacking CD45, without being able to identify them it is impossible to predict whether they are a cause for any differences in the CD45 knockout cells. A future study should involve excision from a 2-D gel followed by mass spectrometry to determine their identities.

As predicted, several SFK were hyperphosphorylated in CD45$^{-/-}$ BMDC even before TLR stimulation. Since CD45 can dephosphorylate both the activating and the inhibitory tyrsosine residues on the SFK (94, 186), simply looking at the tyrosine phosphorylation status is not indicative of the activation state of the kinase. Since Lyn has previously been shown to participate in LPS signaling (114, 152), the activation of Lyn following TLR4 ligation was assessed in the CD45$^{-/-}$ BMDCs. Tyrosine 507, corresponding the inhibitory site was constitutively hyperphosphorylated suggesting that Lyn is inactive in these cells. As previously discussed, the CD45$^{-/-}$ phenotype does not match the observed effects in Lyn$^{-/-}$ BMDC (107) likely due to the fact that CD45 is a master regulator of several kinases. Further research into the activation profiles of the rest of the SFK family members upon TLR stimulation would provide more insight.

Not surprisingly, the activation of p42/44, p38, JNK and NF-κB upon TLR4 ligation were not altered in the absence of CD45. The triple SFK knockout of Lyn/Hck/Fgr also did not show any defects in signaling directly downstream of TLR4 (153) and more recently Smolinska *et al.*, showed that chemical inhibition of SFKs by PP2 also had no effect on the activation of these molecules (144). Of interest, the latter study did find a delay in the formation of the AP-1: DNA complex, as assessed by EMSA, even though activation of JNK was unaffected. This result is similar to that obtained by Napolitani *et al*., where they also observed an effect of PP2 on AP-1 (113). In this study, AP-1 was not assessed with the rationale that JNK activation was normal but given the latest paper by Smolinska *et al*., this should be addressed in future work.

As no effect on canonical TLR signaling was observed in CD45$^{-/-}$ BMDCs, logic dictated looking outside the box at SFK substrates. Two SFK substrates, Cbl and DOK2,

which are negative regulators of TLR4 signaling, showed impaired activation in the absence of CD45. The dysregulation of these negative regulators provides some evidence that fits with the hypothesis that MyD88-dependent signaling is increased in $CD45^{-/-}$ cells. More intriguingly, the TLRs seem to be hyperphosphorylated. The function of TLR tyrosine phosphorylation is only beginning to come to the fore but it already seems as though the SFK are involved in this process (145, 148). The fact that Lyn appears to be inactive in $CD45^{-/-}$ BMDC suggests either that another SFK family member is upregulated or overcompensating for the loss of Lyn or perhaps that CD45 itself may play a role in dephosphorylating the TLR itself.

6.4 Loss of CD45 in innate cells has functional consequences

Cytokine secretion by activated DCs provides an important avenue of communication to other immune cells. Improper or imbalanced cytokine secretion can lead to allergies or autoimmunity. $CD45^{-/-}$ BMDCs activated with LPS were less efficient at priming IFNγ release from NK cells. IFNγ release from NK cells is largely dependent on IL-12. However, other cytokines such as IL-15 and IL-18 (187) have also been recently identified as contributing factors and a contact-dependent mechanism also seems to contribute (22) although this may be related to the trans-presentation of IL-15 by the DCs. Because the decrease in IFNγ from the $CD45^{-/-}$ BMDC: NK cell cocultures only occurred at high LPS concentrations, it is likely that this effect was not solely due to a decrease in IL-12 but is also potentially related to another factor like IL-15. Future experiments should address this, as well as look at the cytolytic activity of NK cells activated by $CD45^{-/-}$ BMDC. $CD45^{-/-}$ LPS-

activated BMDCs do not produce as high levels of type I IFN, which has been shown to be important for conferring cytotoxic effector functions on NK cells (22). Therefore it would be interesting to see if the decreased IFNβ in these cells has a functional effect on NK cell activation.

T cell activation, as assessed by IL-2 production was differentially affected by the loss of CD45 in TLR activated BMDCs. When LPS was used as an activation stimulus, no effect was observed on IL-2 production but IFNγ decreased at all peptide concentrations. For CpG, IL-2 was profoundly affected at all peptide concentrations but with Pam_3Csk_4, a peptide concentration dependent effect occurred. This suggests that lack of CD45 in BMDCs is able to alter the outcome of T cell stimulation as well as T cell polarization although these results have only been obtained *in vitro*. It would be of great benefit to be able to translate these findings into a model of disease pathogenesis to get a more accurate picture of what the net results of these alterations in T cell activation and IFNγ production are in a more realistic scenario.

With the $CD45^{-/-}$ mice lacking normal T and B cell populations, the experiments possible were limited to innate immune responses. LPS-induced septic shock is a model for bacterial sepsis, which is a persistent problem in hospital ICUs and a disease with a very poor prognosis. TNFα plays a predominant role in mediating the majority of the problems associated with sepsis. The $CD45^{-/-}$ BMDCs showed a decrease in TNFα secretion upon LPS-stimulation and the hypothesis was that they would show resistance to endotoxic shock. However, as previously discussed, they showed enhanced susceptibility and elevated serum TNFα. One explanation for this was the increase in macrophages found in these mice. As with the $CD8\alpha^+$ DCs, IRF8 guides the lineage decision to produce macrophages. If cytokine

signaling to IRF8 is increased in the absence of CD45, then knocking down IRF8 in $CD45^{-/-}$ bone-marrow progenitors and injecting them back into irradiated recipients should reverse this phenotype. It would also be beneficial to determine the TNFα secretion per cell by flow cytometry to assess if in fact, each macrophage shows a decrease in intracellular TNFα.

6.5 Limitations of this work, future directions and conclusion

This thesis has been able to show a role for CD45 in DC function and has laid the foundation for others to continue. The major drawback inherent in this study is the lack of *in vivo* validation. The $CD45^{-/-}$ mice have altered development of many cell types (T, B, NK and macrophages) making them unsuitable for *in vivo* infection models and T cell priming. Perturbations in that many cell compartments can lead to changes in the cytokine environments of the hematopoietic niches and changes in the development and function of cell populations that are not a direct result of the lack of CD45.

Adoptive transfer experiments are a step in the right direction. However; that system is still artificial since it relies on TCR transgenic mice and injected DCs. The simple process of injection can cause splenic DC maturation (22) as does cluster disruption, which occurs during BMDC harvest (181). Adding to the complexity is the phenomenon of antigen transfer from DCs migrating into the lymph node to resident DCs (188, 189). In an adoptive transfer model, any effect of the injected $CD45^{-/-}$ DCs on T cell activation might then be compensated for by the resident $CD45^{+/+}$ DCs in the recipient animal. One future direction that might be taken to circumvent this problem is use of the CD11c-DTR mice (190), where CD11c+ cells are deleted upon administration of diphtheria toxin to the mice, to selectively ablate the

$CD45^{+/+}$ DCs. However, this process still relies on adoptive transfer of DCs back into the DT-treated animals but all antigen presentation and T cell activation would be accomplished by the $CD45^{-/-}$ transferred DCs. The ideal scenario would be the creation of a DC-specific CD45 knockout mouse to further extend upon the foundation work outlined in this thesis.

Chapter 7: References

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